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FOREWORD

I am pleased to put into the hands of readers Volume-5; Issue-2: Mar-Apr 2020 of **"International Journal of Environment, Agriculture and Biotechnology (IJEAB) (ISSN: 2456-1878)",** an international journal which publishes peer reviewed quality research papers on a wide variety of topics related to **Environment, Agriculture and Biotechnology**. Looking to the keen interest shown by the authors and readers, the editorial board has decided to release issue with DOI (Digital Object Identifier) from CrossRef also, now using DOI paper of the author is available to the many libraries. This will motivate authors for quick publication of their research papers. Even with these changes our objective remains the same, that is, to encourage young researchers and academicians to think innovatively and share their research findings with others for the betterment of mankind.

I thank all the authors of the research papers for contributing their scholarly articles. Despite many challenges, the entire editorial board has worked tirelessly and helped me to bring out this issue of the journal well in time. They all deserve my heartfelt thanks.

Finally, I hope the readers will make good use of this valuable research material and continue to contribute their research finding for publication in this journal. Constructive comments and suggestions from our readers are welcome for further improvement of the quality and usefulness of the journal.

With warm regards.

Editor-in-Chief
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Agronomic Characters and Chemical composition of Sri Lankan Novel Red Pericarp Rice (*Oryza sativa L.*) Variety

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Abstract— *Sri Lanka is a rich treasurable land of traditional rice cultivation and rice is the primary staple food in Sri Lanka. There are many unpopular rice varieties which have been derived from traditional rice varieties and their nutritional values are under investigation. One such novelSri Lankan red pericarp rice variety known as Gurupiya is focused on this study. Agronomic characters of novel rice variety was evaluated according to IRRI 1998. Standard methods of AOAC nutritional guidelines were used to obtain the proximate composition of Gurupiya rice variety (GPRV). AOCS methods were used to analyze oil profile. Fatty acid profile was determined by using GCMS. The results showed that crude protein, crude fiber, crude fat and carbohydrate contents were12.0±0.1 %, 1.5±0.1 %, 2.6±0.4 %, 75.9±0.1 % respectively. Calorie content was 375.0 kcal/100g. According to the results, GPRV have high Zn content (4.82 mg/100 g). Fatty acid composition of the GPRV revealed that more than half of the total fatty acids were made up of unsaturated fatty acids (USFA) which are considered as important for good health. Results showed that iodine value (IV), acid value (AV), saponification value (SV) and peroxide value (PV) of GPRV were 72.1 ± 3.5 g I_2/100 g, 21.4 ± 2.7 mg KOH/g, 202.13 ± 2.5 mg KOH/g and 18.6± 1.1 meq O_2/kg, respectively. The results reveal that novel GPRV shows high nutritional values than that of reported for the improved rice varieties. Therefore, consumption of GPRV will aid in the improvement of health condition.*

Keywords— ***Traditional rice, proximate, fatty acid, oil, minerals.***

I. INTRODUCTION

Rice is the major staple food in Sri Lanka. It is available in over 5000 varieties all over the world [1]. In ancient times, about 2000 conserved different traditional rice varieties known to have existed in Sri Lanka[2]. Within last few years, traditional rice is gradually making an increment of consumption mainly due to the global awareness on the benefits of consuming organic foods and the drawbacks of using chemical fertilizers and pesticides. The specialties of Sri Lankan traditional rice varieties can be highlighted as high nutritional value, different texture, appearance, aroma and taste compared to that of improved rice varieties[2]. The major nutrients of carbohydrate (32.1 g/100 g grain), protein (2.6 g/100 g grain), fat (1.1 g/100 g grain), and fiber (0.8 g/100 g grain) are consisted in the rice grain and its bran [3]. Health benefits of rice are mainly due to the activities of phytochemicals such as phenolic compounds. Sri Lankan traditional rice varieties of Kaluheenati (KH), Pokkali (PK), Gurusinghe wee (GW), Kahawanu (KW), Sudumurunga(SM) and Unakola samba (US) provide high nutritional value which is free from heavy metals compared to improved rice [4]. Both parboiled and un-parboiled Pokkali rice variety has been proven that it is a good dietary supplement for iron deficiency and for pregnant mothers due to the significantly high iron content [5]. Studies on Sri Lankan traditional and improved rice varieties have shown medium to high amylose content (57-73). The high amylose content of traditional rice varieties corresponds to the lower glycemic index (GI) values and it resulted in decreased insulin responses [6, 7]. Among them Wedaheenati rice exhibited the low GI (57) while the highest GI was reported in improved Bg 406 variety (73) [8]. Quite a few studies have linked GI with not only the rice but also the rice products such as porridges, curries and cereal products. Parboiled rice with *Amaranthus* leaf curry was classified as low GI (47) meal [9] while plain white rice have reported a relatively higher GI value (50-92) [10]. Some parboiled varieties of traditional red rice as well as Bg 350 improved variety have the low GI values

(56-73) compared to some of the un-parboiled red rice and white rice. Research findings have highlighted that red parboiled varieties of rice and Bg 350 can be recommended for patients with diabetes[11]. Previous studies have been provided evidence that the brans of Sri Lankan traditional rice varieties of Masuran, Dik Wee, GodaHeeneti and Sudu Heeneti have great antioxidant properties as well as oxidative stress associated chronic diseases can be prevented by consuming these rice [12]. *In vitro*glycation reversing and anti-glycation activity of Sri Lankan rice bran of 23 traditional as well as 12 improved (both red and white) rice varieties have been evaluated. Significantly high antiamylase and anti-glycation activities were observed for bran extracts of traditional red rice varieties, Masuran, Sudu Heeneti, Dik Wee and GodaHeeneti, compared to that of improved red rice varieties[13].One of the most important investigations illustrated the potential anti- cancer activity of rice bran of four Sri Lankan traditional varieties (GodaHeeneti, Sudu Heeneti, Dik Wee and Sudu Heeneti) against human lung cancer (NCI-H460) and cervical cancer (HeLa) cell lines. From the point of view of growth inhibition and cytotoxicity, it is becoming apparent that some of the Sri Lankan traditional rice varieties possess promising ability for management of cancer [14].

Therefore, studying the chemical compositions of the novel rice which has never seen before in the national collection of farmers has become a timely need for finding solutions in ways to overcome malnutrition and health problems. The main objective of this study was to determination of agronomic characteristics and chemical parameters (nutritional values, minerals, fatty-acid profile and rice oil) of novel rice variety "Gurupiya" which is known to be derived from traditional rice.

II. MATERIALS AND METHODS

2.1 Determination of agronomic parameters

The agronomic parameters were determined according to the method of Standard Evaluation System for Rice[15]. Plants which have grown in "Yala" season were taken in to account. Plant height (cm), leaf blade length (cm), leaf blade width (cm), number of tillers per plant, number of internodes per plant, panicle length (cm), 100 grain weight (g), seed length (mm) and seed width (mm) were evaluated in this study. The previously reported traditional rice variety, KaluHeenati was used as the reference recommended rice cultivar for analyzing the agronomic parameters. [16]

2.2 Determination of chemical parameters

2.2.1 Preparation of rice samples

Raw paddy was de hulled (Satake THU 35B), ground and passed through a 500 µm sieve to obtain a homogeneous fine powder. Freshly prepared samples were used to analyze chemical parameters.

2.2.2 Proximate analysis

Uniform fractions of rice flour were used to analyze moisture, protein (%N x 6.25), fat, ash, fiber and carbohydrate by the methods of AOAC (2002).

2.2.3 Calorie content

Calorie content (kcal/ 100 g) was calculated by use of specific energy factors of 4:9:4 for proteins, fat and carbohydrates, respectively.

2.2.4 Analysis of Minerals

An amount of 5.0 g of the ground rice sample was taken to a crucible and ashing procedure was carried out at 550°C in a muffle furnace (n = 3). Then, about 10 drops of conc. Hydrochloric acid were added to the ashed sample and mixed well. It was filtered into a 250 ml volumetric flask and was topped up to the mark using distilled water. The final solution was used to analyze minerals (K, Na, Zn, Fe) using atomic absorption spectrometry (Thermo scientific, iCE3000 Series AAS).

2.2.5 Analysis of fatty acid profile

Fatty Acid Methyl Esters (FAMEs) were prepared by according to the ISO 5509-1978 (E) method. Then FAMEs were identified on GC model-7890 A, Agilent technologies equipped with Mass Spectrometer (MS) model-5975 C inert XL EI/CI MSD with triple-axis detector.

2.2.6 Analysis of rice oil

Saponification value was determined according to the A. O. C. S. Official Method Cd 3-25, 1999. Iodine value was determined (Wijs method) in accordance to the A. O. C. S. Official Method Cd 1-25, 1999. Peroxide value was determined according to the A. O. C. S. Official Method, Cd 8b, 1999. Acid value was determined according to the A. O. C. S. Official Method, Cd 3a-63, 1999.

III. RESULTS AND DISCUSSION

3.1 Determination of agronomic parameters

The average results of the measured agronomic parameters are given in the table 01. Age of the plants was 90 days when measuring the agronomic parameters.

Table.1. Average values of each trait of Gurupiya rice cultivar

Plant height (cm)	110.4±0.7
Leaf blade length (cm)	50.2±0.3
Leaf blade width (cm)	1.2±0.03

Number of tillers per plant	4
Number of internodes per plant	4
Panicle length (cm)	21.8±0.2
Seed length (mm)	8.0±0.1
Seed width (mm)	3.2±0.04
100 grain weight (g) (before de hulled)	3.1±0.03

The novel GPRV has red color pericarp. The weight of 100 seeds of GPRV was 3.1±0.03g. The highest value for 100 seeds weight was recorded for Galpawee (3.10g) and the lowest value recorded for that was Mahasudu wee (1.25g). A well-known traditional rice variety, KaluHeenati was selected as a reference rice cultivar throughout this study and it has the weight of 2.33 g/100 seeds. The average seed length of GPRV was 8.0±0.1 mm and the average seed width was 3.2±0.04 mm. These values were in the similar range of KaluHeenati, which were 8.15 mm long and 2.86 mm width. The longest length of seeds was found in the Thanthiribalan (9.12 mm) and the shortest was in the Hathiel variety (5.26mm). The highest seed width of 3.53 mm was reported for cultivar Podihatatha and the lowest seed width of 2.26 mm was recorded for Rathran wee [16]. According to the previous studies, about 65% of the evaluated traditional rice cultivars had reported the average plant heights in the range of 60 cm – 100 cm. The tallest rice cultivar, Podihatatha reported the height of 198 cm while KaluHeenati reported 86.8 cm of height[16]. Furthermore, the leaf blade length of GPRV showed in between value of 50.2±0.3 cm while Podihatatha rice cultivar was reported to have the highest value of 94.2 cm and KaluHeenati displayed as 40.9 cm. The lowest leaf blade length was found in Hatheil rice cultivar of 8.7 cm [16]. Moreover, the leaf blade width of GPRV (1.2 cm) is higher than that of Kaluheenai (0.9 cm). Kotanavalu cultivar had the broadest leaf blade width of 1.6 cm, whereas Mahakuruwee had the narrowest leaf blade of 0.6 cm. According to the results, GPRV has produced 4 tillers per plant and that value is higher than the tillers per KaluHeenati plant, which was the value of 2.6. Previous research has indicated that 55% of the rice cultivars produced less than 5 tillers per plant among the evaluated twenty cultivars [16]. Particularly, the panicle length of GPRV (21.8±0.02cm) is in close agreement with that of KaluHeenati , which is 21.5 cm. The longest panicle length was reported by Kahata wee (29 cm) where the shortest panicle was found in Rathran wee (15.1cm) [16].

3.2 Determination of chemical parameters

The current study found that moisture content of novel rice variety GPRV was 7.5±0.1 % and this value demonstrated the low moisture content compared to the other reported values for traditional rice varieties [4]. In a study which set out to determine proximate composition of rice, it was revealed that both Pokkali and Unakola Samba equally possess the high amount of moisture (11.9%) content [4].This low amount of moisture content of GPRV suggests the high capacity of storage period of Gurupiya cultivar. The crude protein content of GPRV was 12.0±0.1 %, which indicates the higher amount of protein content compared to the previously studied six traditional rice varieties and improved varieties. Reported protein contents of Kaluheenati, Pokkali, Gurusinghe wee, Kahawanu, Sudumurunga and Unakola samba were in the range of 9.7±0.3 % - 11.0±0.4 % [4], whereas improved Bg rice varieties were in the range of 6%-11%.[17] However, studies have found that significant differences between plant and animal protein sources, mostly with cereal proteins such as rice because of their low lysine content.[18]A number of studies have shown the benefits of plant proteins over animal proteins for lowering blood pressure[19] and risk of type 2 diabates[20]. Therefore, this novel GPRV is a good source of plant protein which corresponds to health benefits. The crude fat content of the GPRV was 2.6±0.4 % which is similar to the value of KaluHeenati.[4] Studies on chemical parameters of six traditional rice varieties have reported that their fat content was in the range of 2.3% - 2.9%.[4] Crude fiber content and ash content of GPRV was significantly high (1.5±0.1 % and 2.0±0.03 % respectively) compared to that of previously reported traditional rice.[4] High fiber content of diet leads to several health benefits such as reducing gastrointestinal disorders including gastroesophageal reflux disease, duodenal ulcer, diverticulitis, constipation and hemorrhoids.[21] The total carbohydrate content of GPRV was calculated using reduction method and lies in the range of 75.9±0.1 %. This study proves that novel rice variety shows higher percentages of proteins, fat, fiber, ash and lower percentages of carbohydrates compared to widely consumed improved rice varieties in Sri Lanka.[22] Caloric value of GPRV was 375.0 Kcal/100g. Calorie contents of previously reported traditional rice varieties were in the range of 352.3-372.8 Kcal/100 g. Among those values 372.8 Kcal/100 g was reported for KaluHeenati.[4] This high energy value of GPRV implies its high value to the body as a fuel for metabolic processes.

According to the mineral analysis, GPRV possess high amount of Zn (4.8±0.5 mg/100 g) compared to the previously reported data [5]. GPRV is a good solution as a diet with high Zn content for people having zinc deficiency because studies have found that about 25% of the world's population is at risk of zinc deficiency.[23] The Na content and K content of the GPRV were 8.2±0.5 mg/100 g and 5.4±0.4 mg/100 g respectively.

Fatty acid composition of the rice varieties depend on the genotype, seed maturity, climatic condition, growth and interaction between these factors [24]. The fatty acid profile of GPRV is presented in Table 2.

Table.2. Fatty acid profile of GPRV

Fatty Acids								
16:0	16:1n-7	18:0	18:1n-9	20:0	20:1n-7	22:0	24:0	26:0
20.0±0.2	0.75±0.1	4.77±0.2	63.55±0.1	2.75±0.1	2.46±0.1	2.85±0.2	1.28±0.1	1.50±0.1

*Expressed as % of total fatty acids

The most prominent fatty acid is oleic acid (18:1n-9) and second highest is palmitic acid (16:0) presented in this GPRV. The percentage of total unsaturated fatty acids (USFA) in the GPRV was 66.75±0.2 %. It indicates that more than half of the total fatty acids were made up of USFA which are considered as important for good health. Oleic acid is considered as an essential fatty acid required for human growth and development and must be obtained from the diet and it is included in GPRV as the highest abundant fatty acid (63.55%). Value of the USFA (unsaturated fatty acid) /SFA (saturated fatty acid) ratio was high (2.01). Therefore the GPRV is healthy and safe as manifested in the results. Further, higher contents of monounsaturated fatty acids including oleic acid enhance the stability of oil during cooking [25]. With regards to the current recommendations, there is a requirement of increasing the consumption of USFA and decrease intake of saturated fatty acids, in order to reduce cardiovascular disease risk. Hence, this novel red pericarp GPRV can be recommended as a good source of USFA.

The results of different chemical parameters of rice oil were obtained. These values are in close agreement with those reported in literature for two traditional rice varieties, Rathusooduru and Madathawalu[26]. Iodine value (IV) of GPRV was 72.1 ± 3.5 g I2/100 g. According to Codex standards (1999) the recommended range for refined rice oil is of 90-105 g I_2/100 g. Acid value (AV) is used as an indicator for edibility and suitability for use in industry [27]. According to Codex Alimentarius Commission, AV for crude ice bran oil should be below 50 mg KOH/g of oil. It is a comparative measure of rancidity in terms of free fatty acids which are generally formed during decomposition of oil glycerides. AV of GPRV rice oil was 21.4 ± 2.7 mg KOH/g of oil. The saponification value (SV) of the GPRV found to be 202.13 ± 2.5 mg KOH/g of oil. This value is slightly higher than the upper limit of the codex standard range (180-195 mg KOH/g). High saponification values in KH and KW have the potentials to use in the industries. The shorter the carbon chain, the more acid is liberated per gram of fat hydrolyzed (low SV). Hence it is considered as a measure of the average molecular weight (chain length) of all the fatty acids present. Peroxide value (PV) is used as an indicator of deterioration of oil. Recommended peroxide value should be lower than 10 meq O_2/kg for fresh oils and be lower than 40 meq O_2/kg for crude oil [27]. PV of the GPRV was 18.6± 1.1 meq O_2/kg. Presence of low PV can be further explained in terms of their high antioxidant properties.

IV. CONCLUSION

In conclusion, agronomic characters were evaluated and these parameters are taken into account when this newly found red pericarp rice variety is selected for the commercial cultivation. The weight of 100 seeds of GPRV was 3.02 g.The seed length of GPRV was 8.0 mm and the seed width was 3.2 mm. Basic nutritional values of GPRV were evaluated comparing with popular traditional rice varieties of Sri Lanka such as KaluHeenati, Pokkali and Kahawanu. Crude protein content, crude fat content, crude fiber content and ash content of GPRV was 12.0±0.1 %, 2.6±0.4 %, 1.5±0.1 %, and 2.0±0.03 % respectively. The total carbohydrate content of GPRV was 75.9±0.1 %. The calorie value was 375.0 Kcal/100g. Therefore, GPRV provides a nutritionally complete healthy food which is rich in good, unsaturated fatty acids and minerals compared to improved rice varieties in Sri Lanka as well as in other Asian countries. More specifically, protein requirement can be easily meet by consuming this novel GPRV due its high protein content (12.0±0.1%) Benefits of consuming this type of plant proteins can be emphasized as lowering blood pressure and risk of type 2 diabetes. Moreover, high fiber content of this GPRV (1.5 ±0.1%) leads to several health benefits such as reducing gastrointestinal disorders and avoiding constipation. GPRV is a good solution as a diet with high Zn content for people having zinc deficiency because of the comparatively high amount of Zn content. According to the fatty acid profile, the most prominent fatty acids are palmitic (16:0) and oleic (18:1n-9) presented in this GPRV. Oleic acid is the highest abundant fatty acid (63.55%) which is considered as an essential fatty acid required for human growth and development. Hence, this novel red pericarp GPRV is a good source of USFA which

leads to decrease the intake of saturated fatty acids, in order to reduce cardiovascular disease risk. These primary data can be utilized when this GPRV is screened for further studies.

V. ACKNOWLEDGMENT

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Study of physicochemical parameters and level of cadmium and lead contamination in irrigation water in market garden areas in West Burkina Faso

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Abstract**—** *The market gardening areas of Kodeni and Dogona are among the main market gardening sites in the city of Bobo-Dioulasso (Burkina Faso). On these vegetable perimeters, the forms of water mobilization for irrigation are essentially wells for the Kodeni site and wastewater from sewers for Dogona. In order to assess the physico-chemical quality and the level of cadmium and lead contamination in these waters, samples were taken at different points on each site and outside the site. The method used is based on the sampling of water in 0.5 liter polyethylene bottles, previously rinsed twice with the sample to be taken. The first samples are used to rinse the bottles and perform physical field analyzes which are pH, electrical conductivity (CE), temperature, salinity, turbidity and total dissolved solids (TDS). Each sample was acidified with pure analytical concentrated nitric acid (HNO_3) (0.5 cm^3 in 0.5 liters of water) which was used to determine the metals. Physico-chemical analyzes and the level of cadmium and lead contamination were carried out. The results of these analyzes were processed using hydrochemical techniques (Piper diagram). Principal Component Analysis (PCA) has also been used to highlight the phenomena of mineralization of water in these market gardening areas.*

The results obtained show that these irrigation waters are acidic at the Kodeni site (pH = 5.49) and basic for the Dogona site (pH = 7.95). They are weakly mineralized at the Kodeni site with an average conductivity of 52.56 4µS/ cm and strongly mineralized at the market garden area of Dogona with an average conductivity of 508.4 4µS / cm. The cadmium and lead contents are sometimes higher than those recommended by the WHO (0.01 mg/ L for cadmium and 0.003 mg/ L for lead). The chemical facies give sodium calcium water. The value of the sodium absorption ratio (11.85) of the water at the Dogona site and that of the pH (5.49) at the Kodeni site show that the irrigation water is chemically unsuitable for agricultural use during the dry season.

Keywords**— Bobo-Dioulasso, Kodeni and Dogona market gardening sites, irrigation water, physicochemical quality, heavy metals, dry seasons.**

I. INTRODUCTION

The last decade has seen rapid growth in the populations of cities in developing countries. This demographic explosion associated with advanced urbanization and development subjects populations to difficulties relating to the supply of fresh food products and the availability of

exploitable land (Bremner, 2012) for agriculture. It also results in ever-increasing wastewater flows that surpass current management, treatment and handling capacities (Huibers et *al*., 2001). Added to this is the climatic changes observed in recent years, characterized by the increase in average temperature and the scarcity of rains, which have as a corollary the degradation of the quality and availability of water (Denicola and *al*., 2015). In cities in Africa and Latin America, water scarcity and the food crisis are the major causes behind the use of wastewater and groundwater for irrigation. In sub-Saharan Africa, all the countries of the Sahel strip face a relatively long dry season (Sou, 2009). During this period, the food supply of urban areas is mainly provided by urban and peri-urban agriculture (AUP). Large volumes of wastewater produced in homes, hospitals or industries are discharged into open channels without prior treatment and these waters are often perennial and accessible water resources during the dry season to allow the realization of the agricultural activity (Sou, 2009). The same is true for groundwater, which is difficult to access and most often it dries up in wells and boreholes.

The use of wastewater in urban agriculture overcomes the problem of significant water needs for agriculture, estimated at 70% of withdrawals, or even 95% in some developing countries. On the other hand, in sub-Saharan Africa, it is recognized that the potential of groundwater can further support the development of small and large-scale irrigation and thereby reduce poverty.

However, this wastewater is generally used without prior treatment or with a partial treatment thus promoting contamination of the vegetables. Wastewater contains many pollutants such as suspended solids, pathogenic microorganisms, heavy metals, pesticides, which can make this water unsuitable for irrigation (Khan et *al*., 2013; Gatto et *al*., 2015). Various substances, among which heavy metals such as cadmium (Cd), lead (Pb) see their contents increasing in soils (Braud, 2007). This study was initiated to assess the physico-chemical quality and contamination of irrigation water in market gardening areas in the West of Burkina Faso. It is based on the use of data from hydrochemical parameters and the presence of heavy metals (cadmium and lead) to characterize the level of contamination and the phenomena which are at the origin of water degradation.

II. MATERIALS AND METHOD

2.1. Study areas

The study was carried out in the west of Burkina Faso precisely in the city of Bobo-Dioulasso (04 ° 20'W, 11 ° 06'N, 405 m above sea level). The work took place on the market gardening sites of Dogona and Kodeni. The perimeter of Kodeni (3 ° 55'W, 12 ° 31''N, 449 m) is located at the exit of the town of Bobo-Dioulasso on the Bobo-Banfora axis. As for the Dogona site (3 ° 60''W, 12 ° 38''N, 385 m above sea level), it is located in the heart of the city of Bobo-Dioulasso. According to Fontes and Guinko (1995), Bobo-Dioulasso is located between the 900 and 1100 mm isohyets and characterized by a South Sudanese climate. Seasonally, we have a dry season from November to April and a rainy season from May to October. Average monthly minimum temperatures range from 18 ° C to 25 ° C in May. The location of the study sites is shown in Figure 1.

2.1. Sampling method

Water sampling was carried out during the dry season. It focused on well water for the Kodeni site and surface water for the Dogona perimeter. At each site, we have identified five sampling points. In addition, on each perimeter, a control sample is taken from a water source located upstream. The samples were taken in polyethylene bottles of 0.5 liter capacity, previously rinsed two times, with the sample to be taken. The bottles are filled avoiding the appearance of air bubbles and hermetically closed after acidification of the sample with concentrated analytical pure nitric acid (HNO3) (0.5 cm^3 in 0.5 liters of water). Each bottle has a number on it that identifies the unique sample. All samples were kept in an icebox containing ice and transported to the laboratory the same day.

During sampling, the physical parameters of the water such as pH, electrical conductivity (CE), temperature, salinity, turbidity and total dissolved solids (TDS) were measured in situ. The equipment used in the field consists of a Star 4 pH meter for measuring pH and Eh, a Hach Sension 5 conductivity meter for measuring electrical conductivity (EC) and salinity (Sal).

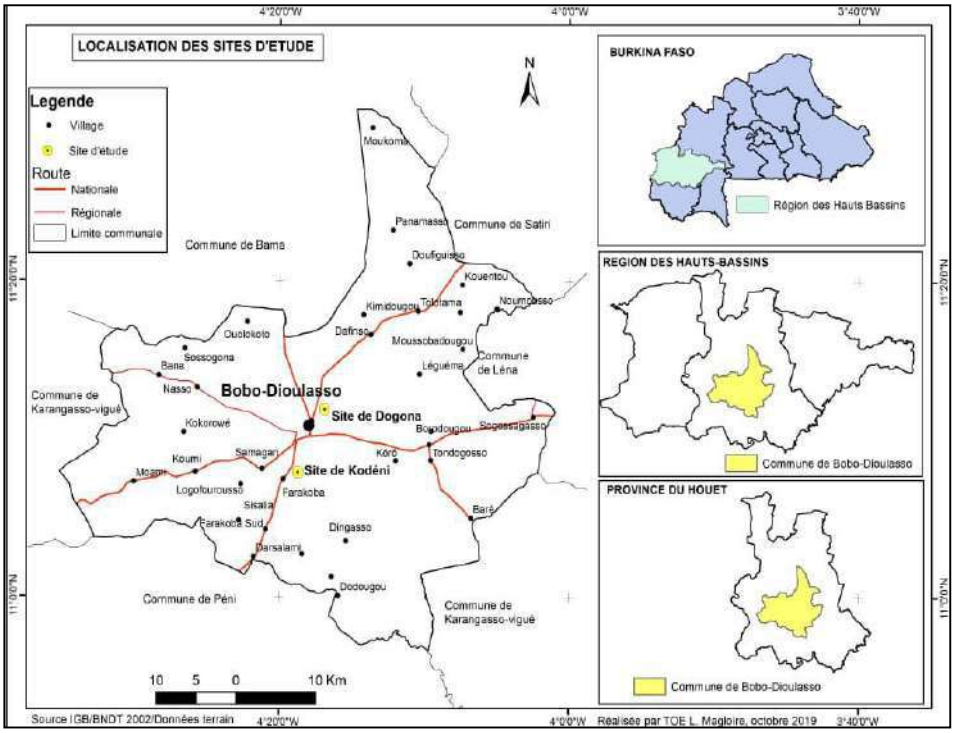

Fig.1. Location of study sites

2.3. Data analysis and processing

Once the samples were sent to the laboratory, we proceeded to determine the chemical parameters using an atomic absorption spectrometer.

The chemical parameters were measured using the Perkin Elmer model AAnalyst 100 brand atomic absorption spectrometer.

The method consists in determining the concentration of metallic elements (alkali metals, alkaline earth metals, transition metals) as well as the metalloids in a sample. These are atomized using a flame supplied with a mixture of gases (air and acetylene). This makes it possible to quantify the elements sought on the order of ppm or ppb.

However, there is no specific preparation to do before the determination because the samples used are already acidified in the field.

- For the determination of the Na^+ and K^+ ions, 8 cm^3 of the acidified sample is taken in test tubes and then added 2 cm^3 of cesium chloride in order to prevent interference. The whole is homogenized with stirring with a magnetic stirrer. Standards of 10 ppm, 20 ppm and 40 ppm are prepared under the same conditions. The reading is made just after calibration.
- For the determination of Ca^{2+} and Mg^{2+} ions, 8 cm^3 of the acidified sample is taken in test tubes and then added 2 cm^3 of lanthanum chloride in order to prevent interference. The whole is homogenized with stirring with a magnetic stirrer. Standards of 10 ppm, 20 ppm and 40 ppm are prepared under the same conditions. The reading is made just after calibration.
- For the determination of the other cations (Cd and Pb), there is no preparation. It is enough to calibrate the device with the establishes of the element to be determined (standard 1 ppm, 2 ppm, 5 ppm, 10 ppm and 20 ppm), serve the acidified samples in test tubes and go to the correct reading after calibration.

The spectrometer is connected to a computer, on which software is installed which allows the handling of the device. All settings are made using the software interface. Following the calibration, a linear calibration curve is obtained, the linearity coefficient of which is 0.9999. After dosing the different metals, processing and validating the results obtained using quality control. The sodium absorption ratio was calculated using the formula of Berrouch (2011).

$$S.A.R = \frac{[Na+]}{\sqrt{([Ca^{2+}] + [Mg^{2+}])/2}}$$

The data collected in the field and in the laboratory were treated using mutlivariate statistical methods coupled with hydrochemical methods. The hydrochemical method required the use of the Piper diagram for the hydrochemical classification of waters.

The statistical approach is based on the use of Principal Component Analysis (PCA) for the study of the phenomena at the origin of water mineralization. The analyzes were carried out on the basis of 12 variables which are: pH, Turbidity (Turb), TDS, Salinity, CE, Na^+, K^+, $Ca^{2+,}$ $Mg^{2+,}$ Cd and Pb.

III. RESULTS AND DISCUSSION

3.1. Results of the physico-chemical study and heavy metal content

The physico-chemical parameters and the cadmium and lead contents of the waters are given in Table 1.

3.1.1. Results of in situ measurements

The physical parameters of the irrigation water at the Kodeni market garden site are shown in Table 1. On analysis of this, it emerges that the water from the Kodeni market garden area is acidic with an average of 5.49 and that of Dogona is basic with an average of 7.95. Kodeni waters have an average conductivity of 52.56 µS/ cm compared to 508.4 µS/ cm for Dogona waters. Dogona waters have the highest conductivities; these waters are highly mineralized. Only Dogona waters have a salinity with an average of 0.32. The waters of Kodeni have a turbidity which varies from 13.61 to 29.88 NTU with an average of 21.43 NTU. As for the Dogona waters, they have a turbidity varying between 58 and 196 NTU with an average of 102.2 NTU. As for TDS, it has an average of 81.8 and 864.64 mg/ L respectively for the waters of Kodeni and Dogona.

3.1.2. Results of chemical parameters of water

The cations studied in the waters of the market gardening perimeters of Kodeni and Dogona are made up of Na^+, K^+, Ca^{2+} and Mg^{2+}. Among these, the most important are the ca2 + ions which represent 64.86% and 53.11 respectively in the waters of Kodeni and Dogona. Next come sodium Na + with 26.61% and 37.78% respectively at Kodeni and Dogona. In Kodeni waters, Mg^{2+} represent 4.99% and K^+ 3.37%. On the other hand, at Dogona, the Mg^+ ions represent 2.51% against 6.58% for the K+ ions. The sodium absorption ratio (SAR) is 1.97 in Kodeni waters and 11.85 in Dogona waters. The hydrochemical classification of water is presented by the triangular diagram of PIPER (Figure 2). Analysis of this diagram shows that the waters of Kodeni and Dogona are mainly calcium and sodium facies.

3.1.3. Heavy metal content results

In this study, cadmium and lead represent the heavy metals analyzed (Table 1). In Kodeni waters, mean values of 0.02 and 0.87 mg/ L are noted respectively for cadmium and lead. These contents are respectively 0.013 and 0.904 mg/ L for cadmium and lead. These different contents are higher than the WHO guide value (0.003 mg/ L for Cd and 0.01 mg/ L for lead).

Table 1 : Physico-chemical parameters and heavy metals of the irrigation water of the market gardening sites of Kodeni and Dogona

Physico-chemical parameters	**Control value**		**Average**		**Minimum**		**Maximum**	
Physical parameters	**Kdn**	**Dgn**	**Kdn**	**Dgn**	**Kdn**	**Dgn**	**Kdn**	**Dgn**
pH	5.5	5.57	5.49	7.95	5.07	7.58	5.96	8.39
T (°C)	31.6	31.6	30.86	36.42	29.7	33.8	31.9	38.3
Ec (µS/cm)	21.6	21.6	52.56	508.4	17.8	339	81.6	712
Sal	0	0	0	0.32	0	0.1	0	0.5
TUR (NTU)	4.6	4.6	21.43	102.2	13.61	58	29.88	196
TDS (mg/L)	22	22	81.8	864.64	19	156	245	852
Chemical parameters (mg/L)								
Ca^{2+}	7.22	7.22	12.48	145.14	10.32	47.18	14.44	220.6
Mg^{2+}	0.11	0.11	0.96	6.87	0.07	6.2	2.02	7.43
Na^+	1.16	1.76	5.12	103.28	2	2.87	8.31	

							29.44	
K^+	0.38	0.38	0.65	17.97	0.16	2.87	1	23.61
SAR			**Kdn = 1.97**		**Dgn = 11.85**			
Heavy metals (mg/L)								
Cd	0	0.126	0.02	0.013	0	0	0.08	0.041
Pb	1.26	1.26	0.87	0.904	0	0	1.57	2.09

Kdn : Kodeni **Dgn** : Dogona

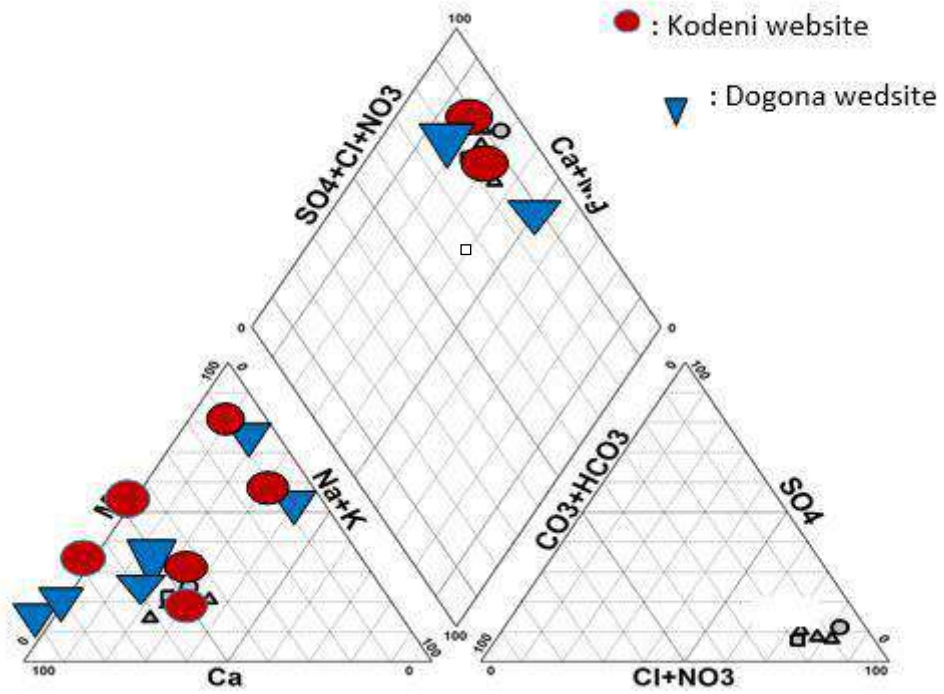

Fig.2: Hydrochemical classification of irrigation water at the Kodeni and Dogona sites

3.1.3. Principal Component Analysis (PCA) for the irrigation water of study sites

- ***Kodeni market garden site***

The eigenvalues of the factors are presented in Table 2. The first three factors represent 92.65% of the variance expressed. These factors include the maximum of the variance expressed and are sufficient to accurately translate the information sought. The correlation matrix between the different variables is presented in Table 3. The correlation coefficient for the conductivity-sodium, conductivity-magnesium, conductivity-TDS, conductivity-salinity, salinity-magnesium, salinity-TDS, TDS-magnesium pairs, TDS-sodium, magnesium-sodium is very strong (r> 0.8). Those for the pH-turbidity, pH-magnesium, temperature-calcium, salinity-sodium, potassium-sodium, patassium-cadmium couples are strong (0.5 <r <0.8). The other correlation coefficients between the measured parameters are medium or low.

The analysis of PCA variables in the factorial plane F1-F2 is presented in Figure 3. This graph highlights two major groupings of the parameters studied in the water withdrawal points. The correlation formed by the axes F1 and F2 gives 71.95% of the total information. The first group which takes into account the pH, turbidity, calcium, temperature, magnesium, sodium, TDS, salinity and potassium and the second, cadmium and lead.

Table 2: Own values of the CPA

	F1	F2	F3	F4
Own values	5.558	3.076	2.485	0.881
Variability (%)	46.315	25.632	20.708	7.345
% cumulative	46.315	71.947	92.655	100.000

Table 3: Correlation matrix between variables

Variables	PH	Ec	T°C	Sal	TUR	TDS	Mg2+	Ca2+	K+	Na+	Cd	Pb
PH	1											
Ec	0,409	1										
T°C	-0,222	0,128	1									
Sal	0,229	**0,979**	0,159	1								
TUR	0,747	0,249	-0,470	0,073	1							
TDS	0,409	**1,000**	0,126	**0,979**	0,249	1						
Mg2+	0,529	**0,944**	0,307	**0,878**	0,359	**0,944**	1					
Ca2+	-0,620	0,183	0,532	0,357	**-0,904**	0,183	0,044	1				
K+	-0,113	0,492	-0,101	0,496	0,405	0,492	0,454	-0,140	1			
Na+	0,478	**0,841**	0,395	0,759	0,431	**0,841**	**0,962**	-0,064	0,552	1		
Cd	-0,663	-0,216	-0,012	-0,143	-0,019	-0,216	-0,238	-0,009	0,704	-0,060	1	
Pb	0,428	-0,434	0,021	-0,516	-0,042	-0,434	-0,315	-0,207	-0,924	-0,371	-0,765	1

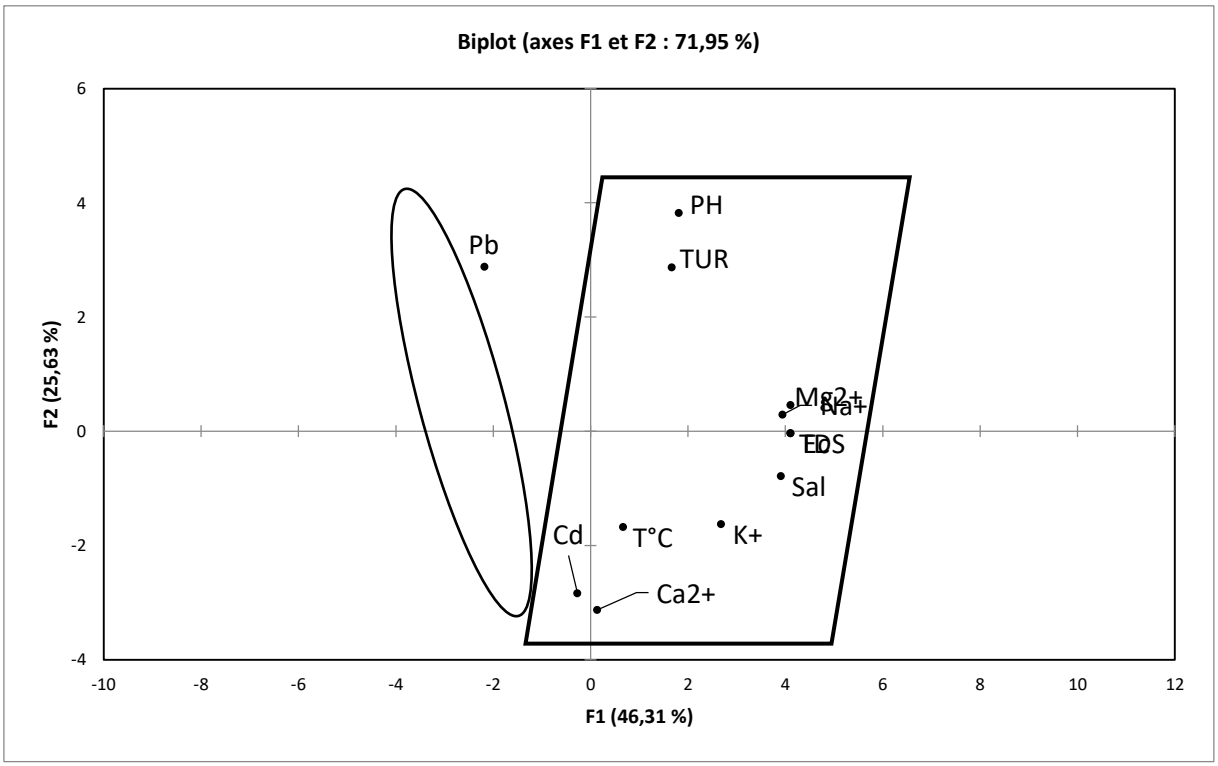

Fig.3: Analysis in the factorial plane F1-F2

➢ ***Dogona market gardening site***

The eigenvalues of the factors are presented in Table 4. The first three factors represent 96.91% of the variance expressed. These factors also include the maximum of the variance expressed. Table 5 presents the correlation matrix between the different variables. The correlation coefficient for the pH-sodium, TDS-conductivity pairs is very high (r> 0.8). Those for the temperature-cadmium, turbidity-magnesium, magnesium-calcium, calcium-cadmium and sodium-lead couples are also strong (0.5 <r <0.8). The other correlation coefficients between the measured parameters are medium or low.

The analysis of the PCA variables in the factorial plane F1-F2 is presented in Figure 4. This graph also highlights two major groupings of the parameters studied in the water withdrawal points. The correlation formed by the axes F1 and F2 gives 77.87% of the total information. The first group takes into account turbidity, calcium, magnesium, patassium and TDS and the second group takes into account temperature, pH, sodium, lead and cadmium.

Table.4: Own values of the CPA

	F1	**F2**	**F3**	**F4**
Own values	4.888	3.677	2.094	0.340
Variability (%)	44.440	33.432	19.038	3.090
% cumulative	44.440	77.872	**96.910**	100.000

Table.5: Correlation matrix between variables

variables	Ph	Ec	T°C	Sal	TURB	TDS	mg2+	Ca2+	K+	Ca2+	Cd	Pb	
pH	1												
Ec	-0,327	1											
T°C	0,794	-0,652	1										
Sal													
TURB	0,067	-0,71	0,135		1								
TDS	-0,334	1	-0,656		-0,71	1							
mg2+	-0,503	-0,589	-0,188		-0,589	-0,583	1						
Ca2+	-0,551	-0,359	-0,059		-0,022	-0,353	0,748	1					
K+	-0,408	0,48	-0,655		0,248	0,481	-0,122	-0,493	1				
Na+	**0,813**	0,272	0,452		-0,402	0,265	**-0,902**	-0,754	-0,143	1			
Cd	0,243	-0,758	0,631		0,115	-0,756	0,476	0,672	**-0,908**	-0,194	1		
Pb	0,197	0,451	0,289		**-0,831**	0,449	-0,733	-0,112	-0,278	0,563	-0,082	1	

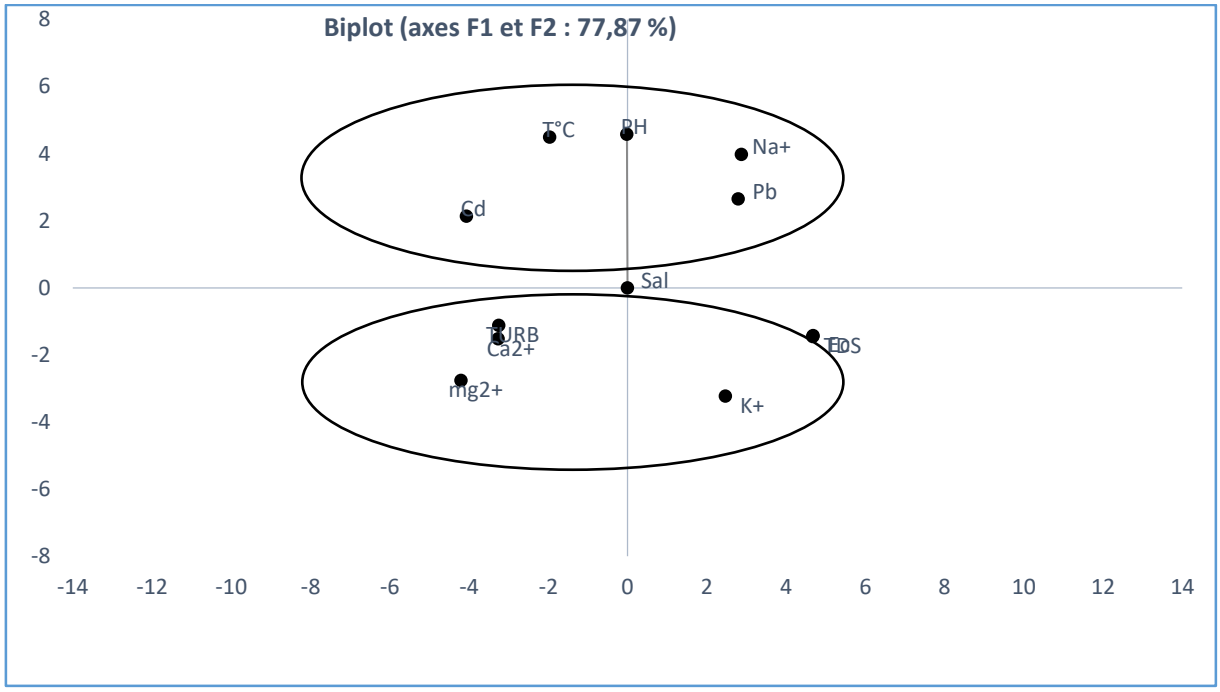

Fig.4: Analysis in the factorial plane F1-F2

3.2. Discussion

3.2.1. Physico-chemical characteristics of the irrigation water of the Kodeni and Dogona market gardening sites

The average temperature of well water (30.86 °C) is close to that of the control well (31.6 °C) and different from that of wastewater (36.42 °C). This difference in temperature of the water from the two sources is explained by the fact that the water from the wells benefits from the shade created by the shrubs present on the site while the wastewater coming from the city is exposed directly to the sun's rays. These observations are similar to those of Pazou et *al*. (2010) which stipulate that heat exchanges with the atmosphere are favored by exposure to the sun's rays. In fact, in dry periods, the ambient temperature increases due to the strong sunshine, which also affects the temperature of the irrigation water.

The pH value 7.95 at the Dogona site is higher compared to that of the waters at the Kodeni site (5.49). This difference could be explained by the effluents of water likely to increase the pH of water such as washing water, dishes, household toilets which are discharged into the gutters. This high pH of wastewater has already been reported by Gemmell and Schmidt (2010) for river water. The low pH of well water is linked to the geological nature and the chemical properties of the soil where the wells were located. Indeed, Matini et *al*. (2009) and Ahoussi et *al*. (2010) have shown that the acidity of water in the humid tropical zone is mainly linked to the decomposition of plant organic matter, with the production of CO_2 in the first layers of the soil. Irrigating soil with such water can contribute to its acidification.

The average conductivity value is 508.4 µS/ cm for wastewater and 52.56 µS/ cm for well water. This difference shows a strong variation in the chemical composition of the waters between the two market gardening sites. These conductivity values indicate a strong mineralization of wastewater especially.

The waters of the Kodeni site have zero salinity; unlike the water at the Dogona site, which has a salinity of 0.32. These results corroborate those of Ahoussi et *al*. (2013) who showed zero value salinity in groundwater in the village of MangouinYrongouin (West of Côte d'Ivoire).

The value of the high turbidity in the wastewater on the market garden area of Dogona reflects the presence of particles in suspension in the water (organic debris, clays, microscopic organisms and urban waste), especially in the dry season there is dimunition of the volume of water.

Their average TDS value is 81.8 mg/ L for the Kodeni site and 864.64 mg/ L for Dogona. The TDS values of the waters change in the same direction as the conductivity. This is explained by the waste discharged by the populations into the canal which serves as a water conduit on the perimeter of Dogona. These results are similar to the work of Senou et *al*. (2016) on the groundwater of a landfill in Bacau (Romania).

The average sodium absorption ratio (SAR) calculated at the two market gardening sites is 1.97 for the Kodeni

market garden site and 11.85 for the Dogona market site, respectively. Irrigation with water from the market gardening site of Kodeni does not present enough risks because water with a SAR between 0 and 6 can generally be used on any type of soil, and this, without risk of accumulation of sodium. However, irrigation water from the Dogona site presents risks of sodium accumulation since water with a SAR of more than 9 should not be used even if the total salt content is relatively low (Benjelloun, 2013).

3.2.2. Contamination of irrigation water from market gardening sites with heavy metals

The results of the analyzes made in the waters of Kodeni and Dogona gave lead concentrations respectively 0.87 mg/ L and 0.903 mg/ L. The concentration of cadmium is 0.02 mg/ L for the Kodeni site and 0.013 mg/ L for Dogona. These concentrations of Cd and Pb in these irrigation waters are higher than the standards recommended by the World Health Organization (WHO) which fixes at 0.01 mg/ L and 0.003 mg/ L the admissible concentrations for the waters of irrigation. Similar results of heavy contamination of irrigation water by heavy metals have already been reported in the market garden area of Houeyiho in southern Benin (Koumolou et *al*.,2013).

This contamination of irrigation water at the Dogona market gardening site by heavy metals is believed to result from the dumping of household waste such as used batteries, cans, plastics, plant debris, dead animals, animal oils. Emptying into the gutters. The contamination of the water at the Kodeni site could be explained by the fact that these wells are left in the open without protection. Pieces of used skips, sachets or boxes of pesticides (or even remnants of pesticides) and other garbage end up in the wells. In addition, the Kodeni site is close to one of the industrial zones of the city of Bobo-Dioulasso, wells by the phenomenon of rainwater runoff can receive various types of contaminant.

IV. CONCLUSION

The data collected during our study made it possible to draw a portrait of the physico-chemical quality and the level of cadmium and lead contamination of the irrigation waters of two market gardening areas in the city of Bobo-Dioulasso (Burkina Faso). In the light of the results obtained at the level of the physico-chemical parameters measured in the waters, there is evidence of a deterioration in the quality of the waters. Indeed, the pH of the water is acceptable for the survival of living organisms, the turbidity and the TDS, as for them remain very high in the waters of the market garden area of Dogona. These very high TDS contents made it possible to maintain the conductivity of the waters at such very high values. The average cadmium and lead contents are higher than those recommended by the WHO. With a SAR greater than 9, irrigation water at the Dogona site poses a risk of sodium accumulation and should not be used for irrigation. Positive correlations (r> 0.8) were also noted between the different parameters.

ACKNOWLEDGEMENT

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Probabilistic modelling of exposure to pesticide residues in foods and tobacco

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***Abstract*— *Background: Several pesticides are currently available on the US market and used on different crops that enter into the human food chain or are used in consumer products, such as food and cigarettes. Some of these pesticides are classified as toxic or carcinogenic to humans. Additionally, little is known about the combined effects of concurrent exposure events. Hence, it is of paramount importance to develop ways to estimate the cumulative, i.e. multi-chemical, exposure to these substances. This study presents a novel approach to estimate the cumulative exposure of the US population to pesticide residues via two routes, foods and tobacco.*

Methods: Cumulative dietary exposure assessment was run using CARES NG® cloud-based software. Calculations were based on the National Health and Nutrition Examination Survey (NHANES) consumption surveys and incorporated the residue monitoring data from the Pesticide Data Program (PDP) database. A two-box model and smoking habits recorded in the NHANES survey were used to calculate the exposure to pesticide residues from smoking cigarettes.

Results: The results of both models were combined to estimate the total aggregate and cumulative exposure. The outcomes show that although the exposure levels are well below the regulatory limits, the exposure among children is higher than among adult smokers on the 99th percentile level. Moreover, the exposure in the adult population is twice as high for smokers than non-smokers. Among the studied pesticides, chlorpyrifos is the pesticide with the highest exposure levels.

Conclusions: The model described in this manuscript provides a new general framework, that can be used to assess the impact of a new pesticide on the population in a broader spectrum than the models typically used for such purpose. To our knowledge, it is the first model that combines the estimation of the pesticide exposure from the diet and smoking cigarettes.

***Keywords*— *pesticide residues, cumulative exposure, CARES NG®, PDP, probabilistic modelling, chlorpyrifos*.**

I. BACKGROUND

Pesticide usage on crops to protect them from insects, rodents and fungi is part of modern life. By their nature, pesticides can be toxic to organisms beyond their target, including humans [1, 2]. Occupationally exposed individuals and those in the immediate area of pesticide application are most at risk of exposure [3]. However, traces of pesticides remaining on the agricultural commodities used as food pose another risk, as many of these chemicals have carcinogenic or toxic classifications [4]. Low dose chronic exposure, through domestic use as well as consuming foods and drinking water, has been observed and linked to possible adverse health effects, such as endocrine disruptions, cancer or negative effects on neurodevelopment and reproductive system [5, 6]. Children are at particular risk of exposure for several reasons, including that children consume more foods relative to their body weight than adults do [7]. Due to these risks related to the dietary exposure to pesticide residues, it is of utmost importance to regularly monitor the exposure levels in the population.

Organophosphate pesticides (OPs) are the most commonly used insecticides globally today [8]. They are applied to many different foods and crops and are also licensed for domestic use in products applied in and around houses, such as insect repellents and indoor/outdoor foggers. Metabolites of OPs were found in 96% of urine sampled in a study on the US

general population, suggesting a widespread chronic low dose exposure [9].

This is most likely caused by the dietary exposure, as increased fruit and vegetable consumption is associated with higher levels of these metabolites [10, 11].

Recent studies have raised concerns regarding low dose chronic OPs exposure and child neurodevelopment outcomes. Increased likelihood of autism or ADHD was associated with the higher concentration of OPs metabolites *in utero* or in early childhood [11, 12]. Another study in the US found that every 522 pounds of OPs applied within 1km of a pregnant woman's home correlates to a loss of two IQ points in her child at age 7 [13]. One of the most toxic organophosphates currently used on the agricultural market is chlorpyrifos. In 2018, the federal court in the US has ordered the Environmental Protection Agency (EPA) to ban chlorpyrifos, due to its potential link to development of learning difficulties in children [14].Although numerous studies relate to dietary exposure to pesticide residues, little is known about the combined effects of concurrent, i.e. multi-source, exposure events [15, 16].

The literature suggests that smoking is another chronic pesticide exposure route. The traces of the pesticides used on tobacco leaves are present in the dried tobacco used in cigarettes, and effectively burnt along with tobacco and other additives to be inhaled by both active and passive smokers [17, 18]. Similar risks are related to smoking marijuana [19], making both tobacco and marijuana two sources of pesticide exposure in the general population that might increase the risk of adverse health-effects. Despite this, there is no model for quantifying the amounts of inhaled pesticides to which the smokers are exposed. Hence, it is of paramount importance to develop ways to estimate the cumulative, i.e. multi-chemical, exposure to the pesticide residues in dietary sources combined with the exposure to pesticide residues that might be inhaled while smoking.

This study aimed to develop a framework that facilitates the estimation and understanding of the cumulative exposure to pesticide residues on the US population. The model presented in this manuscript proposes a novel probabilistic approach to modelling exposure to pesticide residues via two routes, diet and smoking cigarettes. Long-term dietary exposure was calculated with the CARES NG® cloud-based probabilistic software model which facilitates multi-source, multi-route aggregate (for individual chemicals) and cumulative (for multiple chemicals) exposure and risk assessments [20]. The quantities of pesticide residues inhaled while smoking cigarettes were estimated with a two-box model, primarily developed by RIFM (Research Institute of Fragrance Materials) for assessing the exposure to fragrance materials [21]. The framework was applied on a range of foods and pesticides, including some OPs. The main scope of this analysis was to check whether the framework returns realistic exposure estimates when applied to the real-world data. The proposed framework can be a starting point for further and more refined analyses of the cumulative exposure to pesticide coming from various sources and used as a tool not only for getting a better understanding of the current exposure levels, but also for assessing the impact or a need for new pesticides coming onto the market.

II. METHODS

We combined the US National Health and Nutrition Examination Survey (NHANES) data [22] with monitoring data from the Pesticide Data Program (PDP) [23], which provides information about the pesticide residue levels in foods sold on the American market. Five food commodities were chosen as a subset of the total diet: strawberries, tomatoes, lettuce, apples and rice. The dietary exposure assessment was performed with the CARES NG® model. The quantities of pesticide residues inhaled while smoking cigarettes were calculated for the same NHANES subjects

Table 1 NHANES 2005-2010 population summary.

Group	Age range (years)	Gender	Mean body weight [kg]	Subject count	Smokers count	% of smokers
Children	0-18	Males	40.9	5,272	188	3.6%
		Females	38.8	5,155	173	3.3%
Adults	19-85	Males	88.9	6,789	1,673	25%
		Females	75.7	7,457	1,338	18%

using their smoking habits data and the two-box model, used previously for assessing exposure to fragrance materials and developed by RIFM. All data used in this project is publicly available.

Consumption data

What We Eat In America (WWEIA) is a national 2-day food consumption survey, part of the US National Health and Nutrition Examination Survey (NHANES), an annual program conducted by the National Centre for Health Statistics (NCHS) [24]. It combines interviews and medical examinations designed to assess the health and nutritional status of adults and children in the US. The U.S. EPA's Office of Pesticide Programs (OPP) developed the Food Commodity Intake Database (FCID) to translate foods "as eaten", reported in NHANES, to a food commodity basis. FCID uses recipe files to break down all foods into their raw agricultural commodity (RAC) equivalents. WWEIA data is expressed as grams of food commodity consumed per kg body weight per day for over 500 commodities derived from more than 7,000 different foods and beverages. In this study, a merged version of WWEIA/NHANES surveys from 2005 - 2010 was used, containing demographic data for 24,673 subjects, such as body weight, gender, age, two-day food consumption diary based on 24-hour recall data, and smoking habits of each subject including the average number of cigarettes smoked per day in the last 30 days. Each subject had a statistical weight assigned in order to make the sample of the subjects more representative of the total population, increasing the reliability and precision of the results. The NHANES population was divided into 4 age and gender subgroups, as shown in Table 1.

Pesticide residues in foods

Data related to the pesticide residues in strawberries, tomatoes, lettuce, apples and rice was obtained from the Pesticide Data Program (PDP) database [23]. PDP is a national pesticide residue monitoring program and produces the most comprehensive pesticide residue database in the US. It contains the residue data collected annually in the US from 1994 to 2016. In this project, we focused only on data from 2005-2015.

PDP commodity sampling is based on a rigorous statistical design ensuring that the data is reliable for use in the exposure assessments. The pesticides and commodities to be analysed each year are selected based on the US Environmental Protection Agency (EPA) data needs and taking into account types and amounts of food consumed by infants and children. The number of samples collected by each state is apportioned according to that state's population. Samples are randomly chosen and reflect what is typically available to the consumer throughout the year.

Given the variety of pesticides applied to the US crops, the estimation of the exposure levels from all pesticides would not bring meaningful results. Hence, only a few pesticides for each food group and tobacco were analysed. The pesticides with the highest chances to have adverse effects on people's health for each group were chosen, by selecting the most toxic pesticides with the highest numbers of samples recorded in PDP that contained detected pesticide residue. The sold quantities of each pesticide were also checked to avoid analysing pesticides that are rarely used[16]. Selected pesticides per each commodity and an overview of the data obtained from PDP are given in Table 3.

Table 2 NHANES 2005-2010 adults; smoking habits. Range of cigarettes smoked per smoker was 1-95 per person.

			per day	per day	per day
Males	Smokers	1,673	15.69	40	60
	Total population	6,789	3.86	20	40
Females	Smokers	1,338	13.87	30	44
	Total population	7,457	2.66	20	30

Table 3 Pesticides chosen for comparison, food commodities; summary of PDP data, combined for years 2005-2015.

Commodity	Pesticides considered	Number of samples in PDP containing residues	Total number of samples in PDP	Maximum concentration of pesticide detected [ppm]	MRL [ppm]	% of samples from US
Strawberries	Chlorpyrifos	52	3104	0.130	0.2	90%
	Thiamethoxam	259	2367	0.250	0.3	
	Novaluron	199	882	0.390	0.45	
	Pyriproxyfen	26	2583	0.079	0.5	
Tomatoes	Chlorpyrifos	35	2366	0.055	0.5	58%
	Bifenthrin	221	2347	0.110	0.15	
	Azoxystrobin	372	2366	0.059	0.2	
	Pyriproxyfen	81	2366	0.079	0.8	
Lettuce	Chlorpyrifos	18	1403	0.078	1	98%
	Diazinon	93	1275	0.027	0.7	
	Imidacloprid	640	1403	0.190	3.5	
Apples	Chlorpyrifos	30	3116	0.145	0.01	95%
	Diazinon	161	3116	0.210	0.5	
	Imidacloprid	612	3116	0.051	0.5	
Rice	Malathion	16	933	0.043	8	83%
	MGK-264	66	933	1.439	*	
	Piperonyl butoxide	165	933	20.85	*	

***** No MRLs for MGK-264 and piperonyl butoxide in rice were found at the time of the study.

All detected quantities of the pesticides concentrations were below the allowed Maximum Residue Levels (MRLs), which is the highest legally allowed level of a pesticide residue in or on food or feed [4]. The MRLs considered for inclusion in the study presented here are based on availability and knowledge of the participating authors. The data registered by PDP shows that the majority of the agricultural commodities consumed by the US population is of domestic origin.

Pesticide residues in tobacco

Tobacco is not considered to be a food commodity, so the crops are not included in the PDP. The EPA also does not set the maximum residue levels of pesticide residues on tobacco in the US [25]. Information on the typical pesticide residue values that can be measured on tobacco and toxicology limits were searched for in the scientific literature. However, the studies reporting quantities of pesticide residues in tobacco focus on testing the laboratory method used to estimate the residues rather than providing data on the residues that can be typically found on tobacco. Hence, these data cannot be considered suitable for exposure analyses. For the purpose of this study, the typical residue levels in tobacco were approximated with the Guidance Residue Levels (GRLs), provided by CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco)[26].

In this study, 3 organophosphates with the lowest GRL values were included in the analysis: chlorpyrifos, parathion and terbufos, outlined in Table 4.

Table 4 The organophosphates used on tobacco crops included in the analysis.

Pesticide name	GRL [ppm]
Chlorpyrifos	0.5
Parathion	0.06
Terbufos	0.05

CARES NG®

The Cumulative and Aggregate Risk Evaluation System – New Generation (CARES NG®) is a cloud-based software providing probabilistic modelling of exposure and risk assessments [20]. CARES NG® allows one to run aggregate and cumulative exposure assessments using methods following the current US EPA Office of Pesticide Program (OPP) guidelines [27, 28]. The software is available for public use online at caresng.org.

CARES NG® Dietary Model estimates dietary exposure from pesticide residues in food using NHANES subject data and PDP pesticide residue data. The model is capable of estimating the typical dietary patterns of the US population over the different time frames, from acute, single-day to long-term, chronic exposure. In this study, multi-day exposure was of interest. The multi-day assessment uses the entire available residue distribution, by randomly selecting a residue value from the distribution for each consumption event reported in the NHANES survey.

The dietary module incorporates the EPA/OPP guidance for adjusting residue values based on the percentage of crop treated and residue type. The Percentage of Crop treated of each commodity was set to 100% for all RACs, hence according to the guidance all the non-detects should be modified. However, the rules set by the guidance are not applicable in the cumulative assessment, because it is necessary to preserve the co-occurrence of the different pesticides in the analysed food samples. This information is stored in the PDP database by assigning a sample number to each residue data point. Therefore, the rules were not applied, and no changes were made to the input residue data. CARES NG® Dietary Model allows the usage of modification factors that account for increase or decrease in residues in foods or in water due to preparation (i.e. washing, cooking, peeling) or treatment (i.e. filtration, chlorination). Modification factors were also included in the analysis and were set to default values established by the EPA[28].

For cumulative assessment, chlorpyrifos was used as the Index Chemical, providing a point of reference from which the toxic potencies for all chemicals were standardised. For each pesticide, MRLs were used as Points of Departure (POD) and Uncertainty Factor was set to 100 by default as per EPA recommendation [27], to account for inter- and intra-species differences in relation to the toxic effects.

Table 5 Toxicology limits of the chemicals analysed in the dietary assessments.

Chemical Name	CAS number	Point of Departure [ppm] (MRL)	Relative Potency Factor (RPF)
Chlorpyrifos	2921-88-2	0.01	1
Diazinon	333-41-5	0.5	0.020
Imidacloprid	138261-41-3	0.5	0.020
Azoxystrobin	131860-33-8	0.2	0.050
Bifenthrin	82657-04-3	0.15	0.067
Malathion	121-75-5	8	0.001
MGK-264	113-48-4	0.5	0.020
Piperonyl Butoxide	51-03-6	0.5	0.020
Novaluron	116714-46-6	0.45	0.022
Thiamethoxam	153719-23-4	0.3	0.033
Pyriproxyfen	95737-68-1	0.3	0.033

If MRL was different for a pesticide depending on a crop (e.g. chlorpyrifos for strawberries and apples), a minimum value was used as a POD. These values were used to calculate Relative Potency Factors, which the exposure to each chemical was multiplied by in order to determine the Exposure Equivalents in terms of Index Chemical (chlorpyrifos in this study).

Details of pesticides analysed and the parameter values applied are shown in Table 5.

Two-box model for inhalation exposure

An alternative model to assess the inhalation exposure to tobacco from smoking had to be proposed, as the CARES NG® Dietary Model analyses only exposure from pesticide residues in food and drinking water. To the best of our knowledge, no published model estimates the exposure to pesticide residues from smoking tobacco. Additionally, no studies have been published on the amounts of pesticides from tobacco that are directly inhaled while smoking. Therefore, we propose to use the two-box model [21], developed by Research Institute of Fragrance Materials (RIFM) for assessing exposure to fragrance materials in sprays, perfume, scented candles, etc. In the case of sprays as well as smoking, the exposure to the chemical is happening via inhalation route and there is a single source (e.g. perfume/cigarette) that releases the chemical over a certain period of time, therefore the same model can be applied.

The two-box model describes the change of the concentration of the chemical of interest (here, pesticide residue) in two zones, where Zone 1 is contained in Zone 2 (e.g. zone 1 – bathroom, zone 2 – house). The model takes into account number of parameters, e.g. volumes of both zones, mass of the material of interest, air-flow between the two zones and the outside (ventilation). Moreover, the two-box model allows modelling the situation in which the subject moves from Zone 1 to the surrounding Zone 2 after a specified time. In the case of smoking a cigarette, Zone 1 is a "breathing zone" around the smoker's head ($1m^3$) and Zone 2 is a room in which the subject smokes (e.g. a living room), and where the subject stays after smoking the cigarette. For conservative reasons, it was assumed that all the smoking events occurs indoor, i.e. the subjects smoke only within their houses. The subject is in the Zone 1 for the time of smoking a cigarette - 6 minutes - and then moves to Zone 2, where he/she stays for another 40 minutes (these and other parameters were obtained from scientific studies and are listed together with corresponding references in the Supplementary Material [Additional file 1]).

The concentration of the chemical in the air is multiplied by the inhalation rate, giving as a result the inhaled dose of the chemical. This value is an inhaled dose per cigarette, which then can be multiplied by the average number of cigarettes per day (this value is subject-specific and comes directly from NHANES data) to obtain the absolute exposure per day.

The two-box model does not directly incorporate the active inhalation when smoking a cigarette, i.e. "puffing". However, it is presenting a conservative approach, as it uses the inhalation rate recommended by US EPA for high-intensity activity level during smoking and afterwards [29]. Moreover, this model does not take into account using a cigarette filter and assumes that 100% of the pesticide residue present on the tobacco leaf is transferred into the smoke[30]. The impact of the cigarette filter on the inhalation of pesticides residues is however unknown and there exists no scientific evidence that using the filter decreases the exposure levels.

Toxicological information about pesticides used in the smoking exposure assessment is presented in Table 6. RPFs were calculated in the same way as for the dietary assessment, with the Uncertainty Factors set to 100

Table 6 Toxicology limits of the chemicals analysed in the inhalation assessments.

Chemical Name	CAS number	Point of Departure [ppm] (GRL)	Relative Potency Factor (RPF)
Chlorpyrifos	2921-88-2	0.5	1
Parathion	056-38-2	0.06	8.33
Terbufos	13071-79-9	0.05	10

Table 7 Contribution of food groups to the total average diet; NHANES 2005-2015 consumption surveys.

Commodity	Children male*	Children female*	Children % of total diet	Adults male*	Adults female*	Adults % of total diet
Strawberry	0.29	0.27	0.73%	0.08	0.12	0.67%
Tomato	1.03	0.97	2.59%	0.74	0.68	4.78%
Lettuce	0.15	0.18	0.42%	0.21	0.27	1.61%
Apple	2.97	3.13	7.94%	0.44	0.47	3.08%
Rice	0.34	0.35	0.90%	0.30	0.18	1.61%
Rest of diet	35.07	32.28	87.41%	13.55	12.70	88.25%

*Given in g/kg-day

Total assessment

To obtain the cumulative exposure which the US population is subjected to,exposure levels from both foods and tobacco had to be combined. This was possible because both assessments were based on the same list of subjects, i.e. the participants to the NHANES survey. The amounts of pesticides consumed/inhaled were added on a subject level, matching the exposure from consumed foods and smoking by the subject ID.

III. RESULTS

The outcomes are given as both aggregate and cumulative exposure assessment. Cumulative results are expressed in terms of exposure to chlorpyrifos, which is one of the pesticides analysed in this study and was selected as an index chemical, i.e. point of reference from which the toxic potencies of the other pesticides can be standardised.

Dietary exposure

The present study looked only at a limited number of food groups which contribute overall to less than 15% of a typical American diet (based on the NHANES 2005 -2010 population), as shown in Table 7. This means that all t presented in this manuscript represent only a portion of the dietary exposure sources that can affect the US population.

The results of the cumulative dietary assessment are given in Table 8. Chronic Reference Dose (cRfD) is given as a point of comparison. cRfD is a daily oral exposure of a chemical to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime. For chlorpyrifos, this value is equal to 0.3 µg/kg-day [31]. The results do not present any significant difference in the exposure levels between males and females in both children and adults.

The CARES NG® Dietary Model does not output the contribution of each pesticide to the cumulative exposure. However, it is indeed interesting knowing which pesticides represent the main source of exposure and, consequently, of concern. To obtain this information, each pesticide has been analysed separately by running aggregate exposure assessments in CARES NG®. The results obtained from the aggregate assessments are summarized in Table 9. Piperonyl butoxide is the chemical with the highest exposure levels due to a combination of the high occurrence of detected values in

*

Table 8 Cumulative dietary multi-day exposure.

Statistics	Children male*	Children female*	Adults male*	Adults female*
Mean	0.002	0.002	0.001	0.001
P95	0.005	0.004	0.003	0.003
P99	0.027	0.026	0.014	0.013

Chlorpyrifos used as the Index Chemical. All values given in in µg/kg-day.

Table 9 Aggregate dietary multi-day exposure.

Pesticide name	Statistics	Children male*	Children female*	Adults male*	Adults female*
Chlorpyrifos	Mean	0.001	0.001	< 0.001	< 0.001
	P95	0.003	0.003	0.002	0.002
	P99	0.008	0.008	0.004	0.004
Diazinon	Mean	0.004	0.004	0.001	0.001
	P95	< 0.001	< 0.001	< 0.001	< 0.001
	P99	0.055	0.053	0.007	0.009
Piperonyl Butoxide	Mean	0.015	0.015	0.013	0.008
	P95	0.079	0.081	0.061	0.043
	P99	0.189	0.181	0.119	0.084
Thiamethoxam	Mean	0.001	0.001	< 0.001	< 0.001
	P95	< 0.001	< 0.001	< 0.001	< 0.001
	P99	0.012	0.011	0.003	0.005
Novaluron	Mean	0.003	0.003	0.001	0.002
	P95	0.004	0.003	< 0.001	< 0.001
	P99	0.082	0.08	0.028	0.043
Bifenthrin	Mean	0.003	0.003	0.002	0.002
	P95	0.013	0.013	0.007	0.007
	P99	0.03	0.03	0.02	0.019
Azoxystrobin	Mean	0.003	0.003	0.002	0.001
	P95	0.013	0.012	0.007	0.007
	P99	0.028	0.028	0.018	0.017
Imidacloprid	Mean	0.004	0.005	0.002	0.003
	P95	0.02	0.021	0.011	0.014
	P99	0.092	0.095	0.041	0.049
Malathion	Mean	< 0.001	< 0.001	< 0.001	< 0.001
	P95	< 0.001	< 0.001	< 0.001	< 0.001
	P99	0.002	0.002	< 0.001	0.001
MGK-264	Mean	0.002	0.002	0.002	0.001
	P95	0.011	0.011	0.009	0.006
	P99	0.027	0.025	0.017	0.012
Pyriproxyfen	Mean	0.001	0.001	0.001	0.001
	P95	0.005	0.005	0.003	0.002
	P99	0.012	0.011	0.007	0.007

* *All values given in in µg/kg-day.*

Table 10 Contribution of each pesticide to the total cumulative exposure for the P95 consumer

Pesticide name	Children male	Children female	Adults male	Adults female
Chlorpyrifos	43.02%	43.09%	44.07%	48.25%
Diazinon	<0.01%	<0.01%	<0.01%	<0.01%
Piperonyl Butoxide	22.66%	23.27%	26.88%	20.75%
Thiamethoxam	<0.01%	<0.01%	<0.01%	<0.01%
Novaluron	1.26%	0.95%	0.00%	0.00%
Bifenthrin	12.49%	12.51%	10.33%	11.31%
Azoxystrobin	9.32%	8.62%	7.71%	8.44%
Imidacloprid	5.74%	6.03%	4.85%	6.76%
Malathion	<0.01%	<0.01%	<0.01%	<0.01%
MGK-264	3.15%	3.16%	3.97%	2.90%
Pyriproxyfen	2.37%	2.37%	2.18%	1.59%

samples obtained in the PDP study and high concentration of the chemical, significantly higher than those found in the other foods shown in Table 3.

To compare the contribution of each pesticide in the overall exposure, the results had to be converted to the equivalents of the Index Chemical, here chlorpyrifos, using the Relative Potency Factors (see Methodology and Table 5). Using this conversion, the contributions were calculated for the P95 and P99 consumers, but as the results were very similar, only the contributions for the P95 consumer are presented in Table 10.

Piperonyl butoxide contributes to around 25% of the total cumulative exposure on the P95 level for all four subpopulations, being the second biggest contributor after chlorpyrifos. Chlorpyrifos remains the main contributor because its degree of toxicity is higher than the other pesticides under analysis. The third most contributing pesticide is bifenthrin with contribution ranging from to 10% to 13%. The order of the most contributing pesticides was the same for P99 consumers.

Table 10 Cumulative exposure from inhalation, tobacco smoking.

Pesticide name	Statistics	Adults male*	Adults female*
All chemicals, whole population	Mean	0.00093	0.00075
	P95	0.00568	0.00546
	P99	0.01006	0.00919
All chemicals, smokers only	Mean	0.00380	0.00392
	P95	0.00956	0.00933
	P99	0.01559	0.01569

* *Chlorpyrifos used as the Index Chemical.*
All values given in in µg/kg-day.

Smoking exposure

The results of the assessment of the exposure via inhaling cigarette smoke were compared to the No Observable Adverse Effect Level (NOAEL), which has been determined for inhalation of chlorpyrifos to be equal to 100 µg/kg-day by US EPA [31]. The results are presented in Table 11 and they present similar exposure levels for male and female adults, all below the NOAEL values.

Table 12 reports the results obtained from the aggregate assessments for the total US adult population (smokers and nonsmokers), with the chlorpyrifos equivalent values obtained by multiplication of the exposure by the Relative Potency Factors, reported in brackets. Despite lower exposure levels, the exposure equivalent doses of parathion and terbufos are similar to the exposure levels of chlorpyrifos, due to the higher toxicity of these two pesticides.

Table 11 Aggregate exposure and chlorpyrifos equivalent doses from inhalation, tobacco smoking.

Pesticide name	Statistics	Adults male (chlorpyrifos equivalent dose) *	Adults female (chlorpyrifos equivalent dose) *
Chlorpyrifos	Mean	0.00031	0.00025
	P95	0.00188	0.00180
	P99	0.00332	0.00304
Parathion	Mean	0.00004 (0.00033)	0.00003 (0.00025)
	P95	0.00023 (0.00192)	0.00022 (0.00183)
	P99	0.00040 (0.00333)	0.00037 (0.00308)
Terbufos	Mean	0.00003 (0.0003)	0.00003 (0.0003)
	P95	0.00019 (0.0019)	0.00018 (0.0018)
	P99	0.00034 (0.0034)	0.00031 (0.0031)

* *All values given in in µg/kg-day.*

Total exposure

To calculate total cumulative exposure (food and smoking), cumulative food exposure levels were added to cumulative smoking exposure levels. As smoking was considered only regarding the adults, the exposure for children remains unchanged. The outcomes are shown in Table 13.

The exposure to pesticide residues is the highest for the smokers when the mean and P95 values are compared. For the high-consumer (P99) however, the exposure is the highest among the children. This means that children who consume high quantities of food can reach exposure levels that are even higher than those experienced by smokers.

The exposure levels for high consumers at commodity level are given in Table 14. Tomatoes are, among the foods, the commodity that presents the highest exposure levels in all the subpopulations. They are second only to the tobacco exposure levels in the adult population. According to the NHANES 2005-2010 consumption data, tomatoes contribute to 2.6% of the overall diet of children in the US and around 4.8% of the diet of adults. Of the five food commodities considered in this study, tomatoes are the most widely consumed commodity in adults, and the second most consumed for children. High consumption and relatively high number of samples containing pesticide residues recorded in the PDP are the main drivers of this result. The other four commodities show similar exposure levels that are included in the range of 0.001 - 0.002 µg/kg-day.

Table 12 Total cumulative multi-day exposure, food and smoking.

Statistics	Children male*	Children female*	Adults male*	Adults female*	Adults male, smokers only*	Adults female, smokers only*
Mean	0.0020	0.0020	0.0020	0.0017	0.0049	0.0048
P95	0.0050	0.0040	0.0074	0.0068	0.0111	0.0122
P99	0.0270	0.0260	0.0186	0.0169	0.0237	0.0227

* *Chlorpyrifos used as the Index Chemical. All values given in in µg/kg-day.*

Table 13 Exposure per commodity for high consumers (99th percentile).

Food commodity	Children male*	Children female*	Adults male*	Adults female*
Strawberries	0.001	0.002	< 0.001	0.001
Tomatoes	0.014	0.012	0.008	0.006
Lettuce	< 0.001	0.001	0.001	0.002
Apples	0.003	0.002	< 0.001	< 0.001
Rice	0.002	0.002	0.002	0.002
Tobacco	-	-	0.010	0.009

* *All values given in in µg/kg-day.*

IV. DISCUSSION

This study successfully developed a new framework that allows us to assess the cumulative exposure to pesticides from concurrent sources, in this case ingestion of foods and smoked tobacco. Additionally, the output obtained from applying this framework to real-world data presented some interesting results.

The analysis suggests that, at population level, the exposure to pesticide residues is approximately two times higher in children than in adults. Children's exposure levels are higher than adults' even when accounting for smoking in adults. That is due to the fact that children's consumption of foods per unit of body weight is more than twice larger than adults (note that the body weight of children is substantially smaller than adults). According to the NHANES 2005-2015 consumption data, children consume on average 38.5g of food per kg of bodyweight, whereas adults - 14.9g/kg of bodyweight. Children eat, on average, amounts of foods that are similar to the amounts consumed by adults; however, their bodyweight is significantly lower, which results in their relative exposure being much higher than the exposure among adults.

The food commodity contributing to the most exposure to pesticide residues for the high consumer are tomatoes, which contribute to around 2.6% to overall diet of children in the US and around 4.8% of diet of adults. This is followed by apples and rice in children (each contributing to less than 1% of children diet) and by rice in adults (contributing to about 1.6% of the overall adult diet). Another key observation is that children who are high-consumers of foods (at P99 level) can reach exposure levels that are even higher than those experienced by the smokers.

The exposure levels estimated by the CARES NG® dietary model are lower than the cRfD for oral exposure to chlorpyrifos equal to 0.3 µg/kg-day, even at P99 level (0.9% of cRfD for children, 0.47% of cRfD for adults). This means that at least 99% of the US population is exposed to doses of pesticides that are not of concern for their health.

Focusing on adults only, exposure to pesticide residues in tobacco and food is more than twice as high among smokers than non-smokers. On the population level, that is including smokers and non-smokers, smoking cigarettes does not change the overall exposure significantly, due to only about 20% of the adult population being smokers and 100% of the population being food consumers. However, the difference is clear when comparing the results for the whole population and smokers only, indicating how high the additional risk of inhaling pesticide residues is from smoking cigarettes. According to our analysis, the high consumers of cigarettes are exposed to almost 0.016 µg/kg-day of all three pesticides analysed for smoking in this study. To our knowledge, this is the first attempt to quantify the exposure levels to pesticide residues from smoking tobacco.

The two-box model preserves a conservative approach, using Guidance Residue Levels (GRLs) to approximate the real residue levels in tobacco, which are likely to be higher than the actual residue concentrations. The amounts inhaled during smoking cigarettes are lower than the NOAEL (about 0.016% of NOAEL for smokers on P99 level). However, the regulatory bodies that decide on the NOAEL level do not conduct studies on the effect of the pesticides that would be smoked. The pesticides in cigarettes may undergo the process of pyrolysis during smoking [32], meaning that the chemical might change its composition in the high temperatures and be more harmful than non-smoked compounds. Hence any

comparison between the NOAEL and the amounts of pesticides inhaled while smoking should be carried out with caution.

The results also show no significant differences in the cumulative exposure levels between male and female smokers, even though the number of cigarettes for the high percentiles is bigger for men than women. The reason for that is the body weight of men tends to be larger than the body weight of women, so per unit body weight exposure is in the end similar. In this project, we analysed the exposure to pesticide in tobacco only for people who are active smokers, however second-hand smokers are also at risk of being exposed to these chemicals, especially those living with people who smoke cigarettes indoors.

In all scenarios analysed, the cumulative exposure was below the regulatory limits that were considered suitable to this study, namely the No-Observable-Adverse-Effect-Level for the inhalation exposure (tobacco) and the Chronic Reference Dose for the oral exposure (foods). Overall exposure to each of these pesticides might be higher, as the presented study looked only at the specific food groups, contributing overall to less than 15% of a typical American diet (based on the NHANES population), as shown in Table 7 in the Results section. Moreover, only a limited number of pesticides was analysed. It is likely that the US population is exposed to higher quantities of pesticides coming from the rest of the diet. Therefore, this analysis cannot be used to infer any risk for the health of the US population, but it provides a framework to assess exposure to pesticides from different sources by combining the results from multiple models.

A study analysing the total diet of the US population and all the pesticides currently used in the USA would be very complex and adversely helpful in risk analysis. It would be difficult to determine the main factors that contribute to the total cumulative exposure because of the number of pesticides available on the US market and their different degrees of toxicity. Despite its limitation, the results obtained from this exposure analysis present interesting trends that are worth highlighting, especially in the light of previously neglected exposures to pesticides residues from tobacco leaves.

The lack of a publicly available monitoring pesticide residue data on tobacco crops was another hindrance to this study. Using such monitoring data would provide a more refined analysis of the exposure levels among the smokers. Moreover, tobacco is not the only commodity that is smoked by consumers. Marijuana is, to date, legal in 33 states of the US for medical purposes and in 10 for recreational purposes. It has been shown, that pesticide residues in marijuana are directly transferred into the mainstream smoke and as a result inhaled by the smoker [33]. Medical marijuana is often smoked by patients suffering from various health conditions, such as cancer or AIDS, particularly prone to chemical poisoning [33]. Recreational marijuana on the other hand is often smoked without a filter, providing no protection from the pesticide residues to the smokers. More data on the marijuana consumptions and programs monitoring the pesticide levels on marijuana and tobacco leaves would enable to expand the current analysis even further.

V. CONCLUSIONS

This study presents a novel methodology for assessing the exposure to pesticide residues in dietary sources and tobacco. The analysis suggests that although all exposure levels are below the regulatory limits, the exposure among children is higher than exposure among the adults. Moreover, the exposure to pesticide residues in the adult population is twice as high for smokers than non-smokers. Among the 11 pesticides analysed, chlorpyrifos was the pesticide causing the highest exposure levels.

The model described in this manuscript provides a new general framework, that can be used to assess the impact of a new pesticide on the population in a broader spectrum than the models typically used for such purpose. To our knowledge, it is the first model that combines the estimation of the pesticide exposure from the diet and smoking cigarettes. The importance of such tool is even more substantial now with the marijuana becoming legalised in more parts of the world. However, more monitoring data is needed to refine the assessments.

ABBREVIATIONS

CARES NG®: Cumulative and Aggregate Risk Evaluation System Next Generation

CORESTA: Cooperation Centre for Scientific Research Relative to Tobacco

cRfD: Chronic Reference Dose

EPA: Environmental Protection Agency

FCID: Food Commodity Intake Database

GRL: Guidance Residue Level

MRL: Maximum Residue Level

NCHS: National Centre for Health Statistics

NHANES: National Health and Nutrition Examination Survey

NOAEL: No Observed Adverse Effects Level

OPP: Office of Pesticide Programs

OP: Organophosphate pesticide

P95,P99: 95th, 99th percentile

PDP: Pesticide Data Program

POD: Point of Departure

RAC: Raw Agricultural Commodity

RIFM: Research Institute of Fragrance Materials

RPF: Relative Potency Factor

WWEIA: What We Eat In America

Declarations

Availability of data and material

The datasets used in the current study are available online from:

FCID:http://fcid.foodrisk.org/

NHANES:https://www.cdc.gov/nchs/nhanes/index.htm

PDP:https://www.ams.usda.gov/datasets/pdp

Competing interests

LMG consults for Microbide Limited. MGS is employed by Microbide Limited.

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Authors' contributions

LMG participated in the study design, was involved in the interpretation of the results and worked on the manuscript. MGS participated in the study design. All authors read and approved the final manuscript.

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Study on the Diversity of Spiders (Order: Araneae) of Lalbagh Botanical Garden and Tavarekere Park, Bangalore South

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Abstract*—Spiders belonging to Order Araneae are the largest order in the Class Arachnida and aid in natural pest control. This study focuses on the diversity of spiders in Lalbagh Botanical Garden and Tavarekere Park in Bangalore, Karnataka. The survey was conducted at the respective locations from June to December 2019 which is a period of seven months by using methods like point observation and random sampling using trails. During the study, a total of 21 species belonging to 16 genera and 10 families were documented. Family Salticidea was found to have 6 numbers of species which is the highest as compared to the other families. The statistical analyses and diversity indices were calculated for each study sites. These types of surveys are important for the study of the prevalence of the spider population in given habitats and to create a biodiversity database of spider fauna at the respective study site.*

***Keywords*—Diversity, Lalbagh Botanical Garden, Richness, Spider fauna, Tavarekere Park .**

I. INTRODUCTION

India is known to be a mega diverse country for its high diversity of flora and fauna and contributing to this biodiversity is the Order Araneae which comprises of Spider. Since spiders belong to a group of the most diverse organism, it is necessary to study its diversity as it has been previously neglected or ignored (Palem et al., 2016). Besides its population in forest area, their availability should be checked in the urban areas. Also as the amount of deforestation and settlements increases, there is loss in the natural habitat of spiders (Khan et al., 2019).

In India there are about 1,686 spider species found out of the total species of 44,906 recorded throughout the world. They are considered as biological control agents as they help in maintaining the ecological balance in the nature by feeding on the small insects and in return these spiders are being eaten by birds and other bigger insects (Bhattacharya et al., 2017). The body of spiders is divided into two parts comprising the cephalothorax and abdomen where the cephalothorax has 4 pair of legs and the abdomen does not have any segments (Sebastian & Peter, 2017). Spiders can't eat solid food, so they have to liquefy the food by using digestive juices and then consume this liquid food (Dharmaraj et al., 2017).

A total number of 51 species belonging to 42 genera of 16 families were documented at Malavagoppa Village in Shimoga district (Kumari & Shet, 2019) and a 10 families of spiders were recorded in Gulbarga in 2012 (Deshpande& Paul, 2016). The diversity of Mygalomorphae was found that a total of 111 species under 32 genera and 8 families were seen over 17 states and 2 of the union territories (Dhali et al., 2016), 32 species of spiders belonging to 7 families were observed in different habitats of the University of Pune (Wankhade et al., 2012) and in Aloka, a total of 11 genus and 26 species of family Araenidae were spotted (Shirbhate & Shirbhate, 2017). A total of 40 species of spiders were recorded which belonged to 36 genera and 11 families in Nilgiris (Dharmaraj et al., 2017), 46 species belonging to 34 genera of 13 families were observed in Tumkuru University (A. L. et al., 2019) and 98 individuals of 11 different species were observed and studied from 10 localities in the Satpuda Mountain (Magare, S. R., 2017). In Gibbon Wildlife Sanctuary, 95 species of spiders belonging to 56 genera and 18 families were recorded (Chetia & Kalita, 2012), (Oyewole & Oyelade, 2014) found 1824 individuals of 19 different spider families in Nigeria and 26 species of spiders belonging to 10 families were observed near the River Narmada (Shukla et al., 2015). In the Taleigao Plateue 74 species of spiders belonging to 17

families were recorded (Pandit & Pai, 2017), 81 species of spiders under 51 genera from 19 families were documented in Sacred grooves of Odisha (De & Palita, 2018) and (Halarnkar & Pai, 2018) revealed the presence of 29 species in one location and 30 species of spiders in other location.

II. MATERIALS AND METHODS

Study area:

The study area is located at Lalbagh Botanical Garden (12.95°N 77.59°E) and Tavarekere Park (12°55'43"N 77°36'10"E) in Bangalore. The study was carried out from June 2019 to December 2019. The sites are located in the urban area with a good number of vegetation. Study sites are surrounded by human dwellings; and has well maintained garden and open field. Survey was done three or four times a week in the evening hours.

Temperature and humidity was recorded where Lalbagh Botanical Garden showed maximum temperature and Tavarekere Park showed minimum temperature. Humidity ranged from 54% to 85% at both the study sites. The flora found at the study location was in wide range.

Sampling methods:

Random sampling using trails and point observation was done from the selected study sites throughout the year during day (VinodKumari et al., 2017). Collections were done by hand picking, gentle beating on surroundings so as to make the individual pass into the cleared area for better viewing. The collected spiders were photographed using a digital camera (Nikon camera) in live condition identified and then released to their natural habitat (Kumari&Shet, 2019).

Ground Hand collection is the collection of spider specimen from ground to knee level as it helps to collect the spiders which are found visible on the ground, litter, in broken logs, rocks etc (Shirbhate&Shirbhate, 2017).

Aerial Hand collection is the collection of spider specimen from knee level to arm length level where the web-building and free-living spiders are easily spotted on the foliage and stems of living or dead shrubs, high herbs, tree trunks etc (Shirbhate&Shirbhate, 2017).

Identification of species:

The book "Spiders of India" by PA Sebastian and KV Peter was referred for the identification of the recorded spiders. Along with websites like Project Noah and other scientific papers which helped in the identification.

Recording and analyzing data:

A Record book was used to note down the number of species observed along with the date, time and the environmental factors of the sampling area and also the name of the species were recorded. The data obtained was analyzed using PAST software.

III. EQUATIONS

1) Shannon's diversity index, $\mathbf{H = - \sum Pi\ (lnPi)}$

   where Hl= Shannon Index, Pi = Proportion of individuals belonging to the ith species

2) Simpson's value, $\mathbf{D = \sum ni\ (ni-1)\ /N\ (N-1)}$

   whereni = the number of individuals of a species

   N = Total number of all individuals

3) Pielou'sEvenness$\mathbf{Jl = Hl/Hmax}$

   where Hl = Shannon value, Hmax = Maximum Shannon value if all the species were equally abundant.

4) Margalef's index $\mathbf{(R) = S-1/In(N)}$

   where R= Margalef's richness index, S= total of species, N= total no of individual sample

IV. RESULT AND DISCUSSION

The total number of spider specimen observed were 129 which comprises of 21 species and 16 genera at Lalbagh Botanical Garden and Tavarekere Park which belonged to 10 families of Order Araneae (Table 1). The highest species diversity was observed at Tavarekere Park with a total number of 17 species observed (Fig 1). The family Salticidae had the highest diversity in the whole study area with 5 genera and 6 species. It followed by family Tetragnathidae which showed 2 genera and 2 species and family Hersilidae has 1 genera and 1 specie and then family Lycosidae has 2 genera and 4 species (Table 2).

The dominant species observed in the study area was Leucaugedecorata, followed by Hersiliasavignyi. Three species of spiders were only observed once at the study location during the entire study period. These species are Ctenuscochinensis, Opadometafastigata and Rheneflavigera. Seven species were common in all the study location and those were Argiope catenulate, Pardosapseudoannulata, Hersiliasavignyi, Pardosabirmanica, Menemerusbivittatus, Oxyopesjavanus, and Oxyopesbirmanicus (Fig 1).

The temperature and humidity played a major role in favouring the diversity of spiders. The highest temperature recorded was 29˚C in the month of June in Lalbagh Botanical Garden and lowest in the month of September

and December with 25˚C in Tavarekere Park(Fig 3).Lalbagh had the highest humidity with 85% shown in Fig 4.

The diversity indices of spiders in Lalbagh Botanical Garden and Tavarekere Park are shown in table 3. The richness value of spiders was R= 3.662 in Tavarekere Park and R= 2.556 in Lalbagh Botanical Garden. The diversity calculate by Shannon Weiner's index in Lalbagh was H= 2.215 and in Tavarekere it showed H= 2.239 which comparatively higher. The evenness in Lalbagh was E= 0.8331 and Tavarekere E= 0.552. The Simpson indices value was higher in Lalbagh with SIM= 0.8776 value as compared to Tavarekere with SIM= 0.8361 value (Table 3).

Table 1: Total Number Of Specimen Observed

Sr. No	Family	SPECIES	LALBAGH	TAVAREKERE	TOTAL COUNT
1	Aranidae	*Argiopecatenulata*	4	1	5
2	Corinnidea	*Castianeirazetes*	5	0	5
3	Ctenidae	*Ctenuscochinensis*	0	1	1
4	Hersiliidae	*Hersiliasavignyi*	10	13	23
5	Lycosidae	*Lycosamackenziei*	0	2	2
		Lycosatista	4	0	4
		Pardosapseudoannulata	2	1	3
		Pardosabirmanica	7	1	8
6	Nephilidae	*Herenniamultipuncta*	0	5	5
7	Oxyopidae	*Oxyopesbirmanicus*	6	1	7
		Oxyopesjavanus	1	2	3
		Oxyopeslineatipes	0	2	2
8	Pholcidae	*Smeringopuspallidus*	3	0	3
9	Salticidae	*Hasariusadansoni*	0	4	4
		Menemerusbivittatus	7	2	9
		Myrmarachneorientales	0	5	5
		Myrmarachneplataleoides	0	9	9
		Plexippuspaykulli	0	3	3
		RheneFlavigera	0	1	1
10	Tetragnathidae	*Leucauge decorate*	0	26	26
		Opadometafastigata	1	0	1
				Total	129

Table 2: Number of Family, Genera And Species Of Order Araneae

Family	Genera	Species
Araneidae	1	1
Corinnidea	1	1
Ctenidae	1	1
Hersiliidae	1	1
Lycosidae	2	4
Nephilidae	1	1
Oxyopidae	1	3
Pholcidae	1	1
Salticidae	5	6
Tetragnathidae	2	2

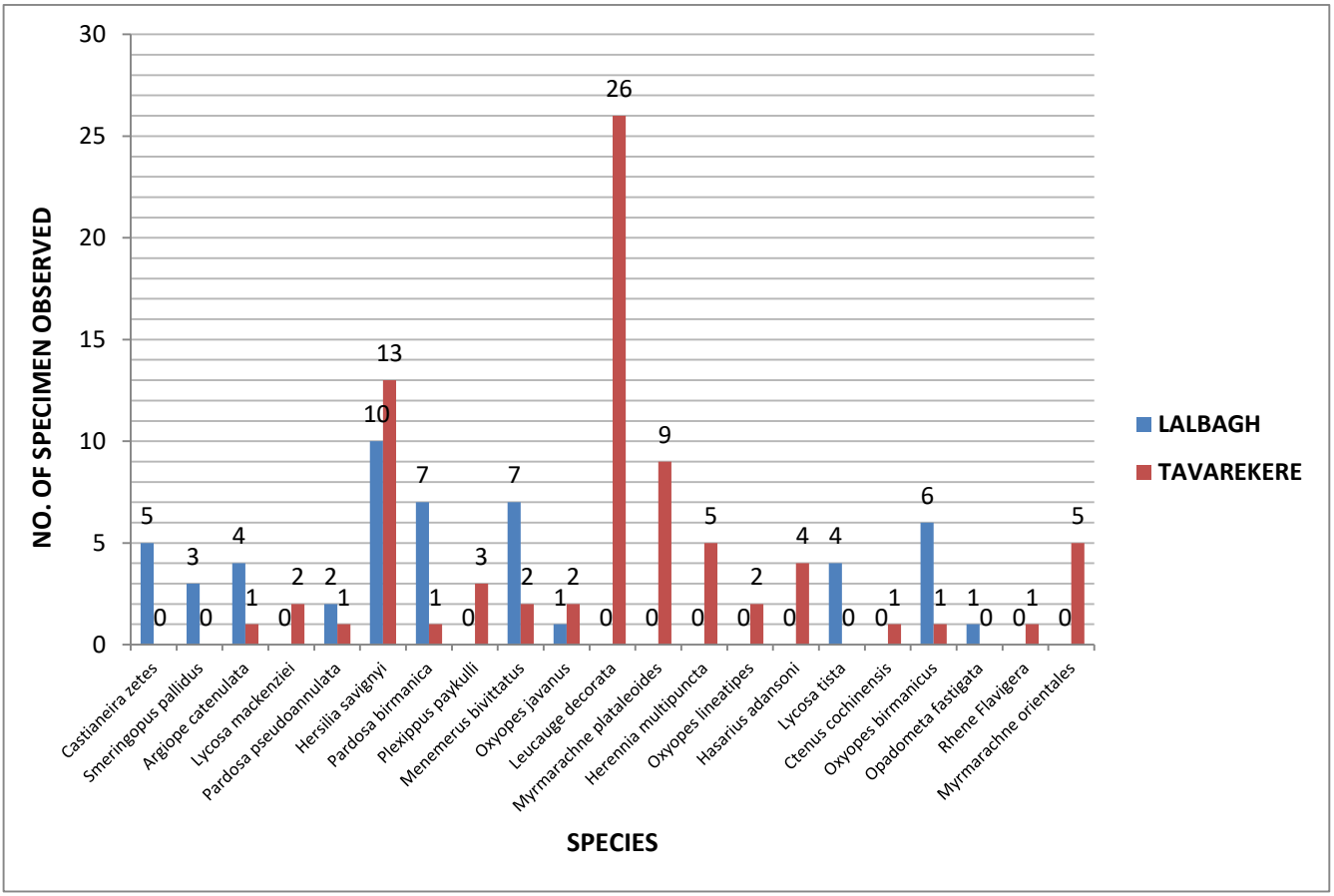

Fig 1: Distribution of Specimen Across Study Area

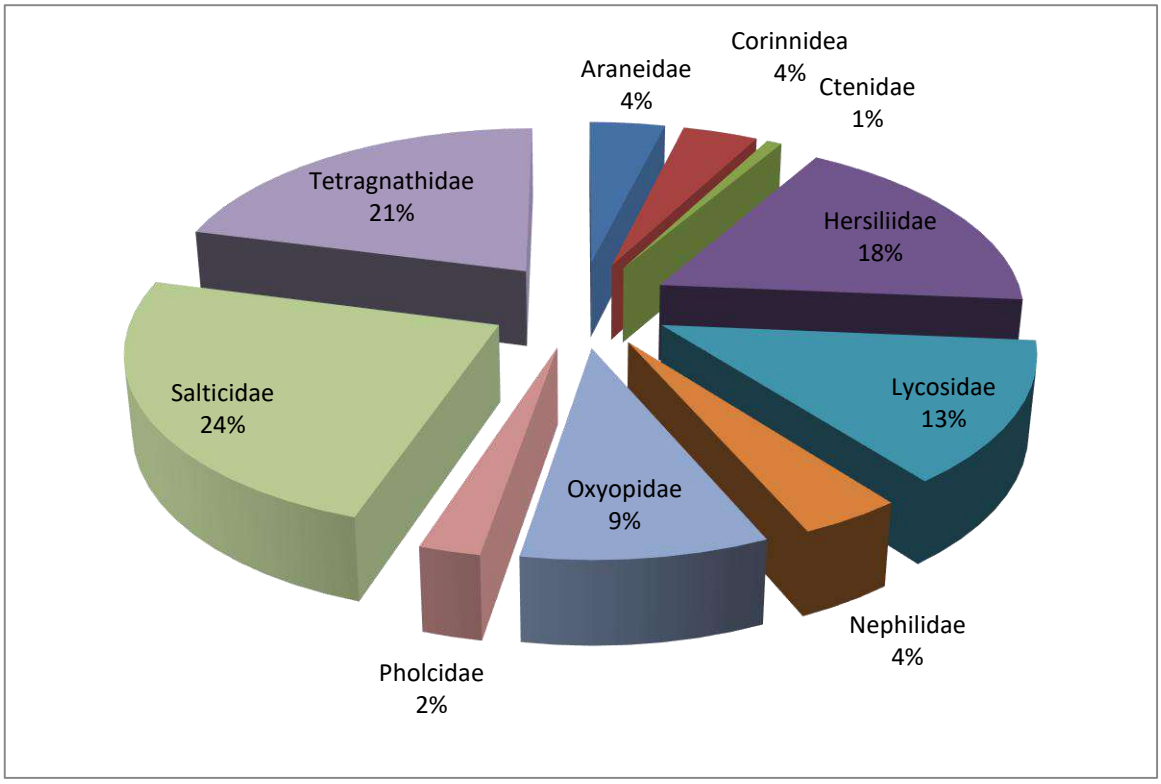

Fig2: Distribution of Araneae Families in the Study Area

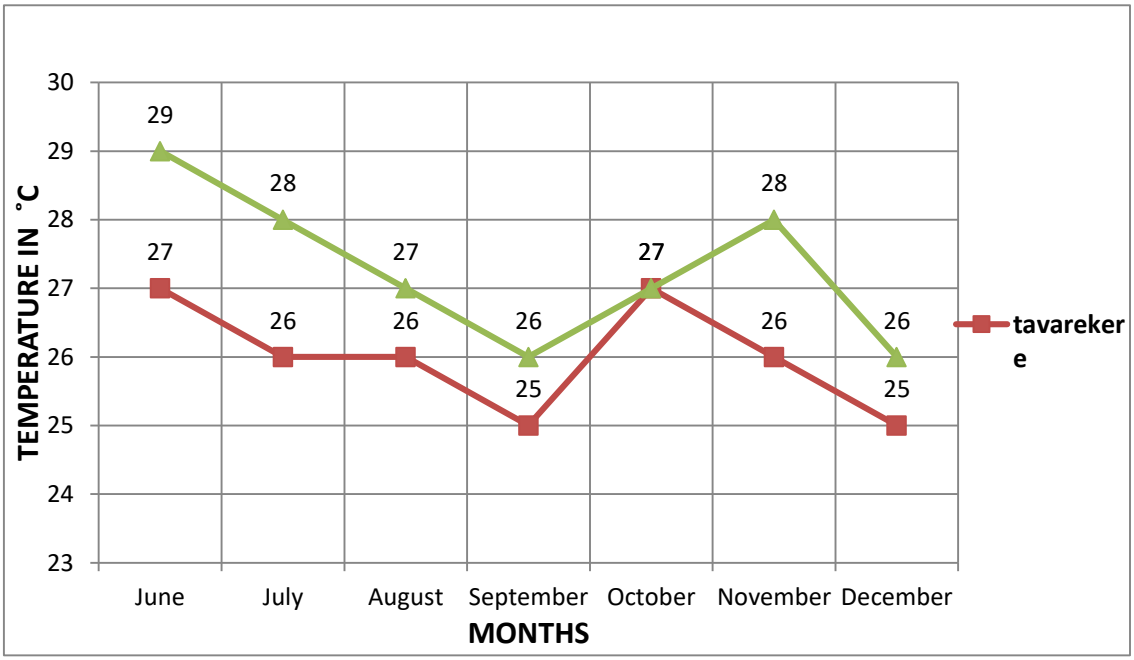

Fig 3: Distribution of Temperature at the Study Area

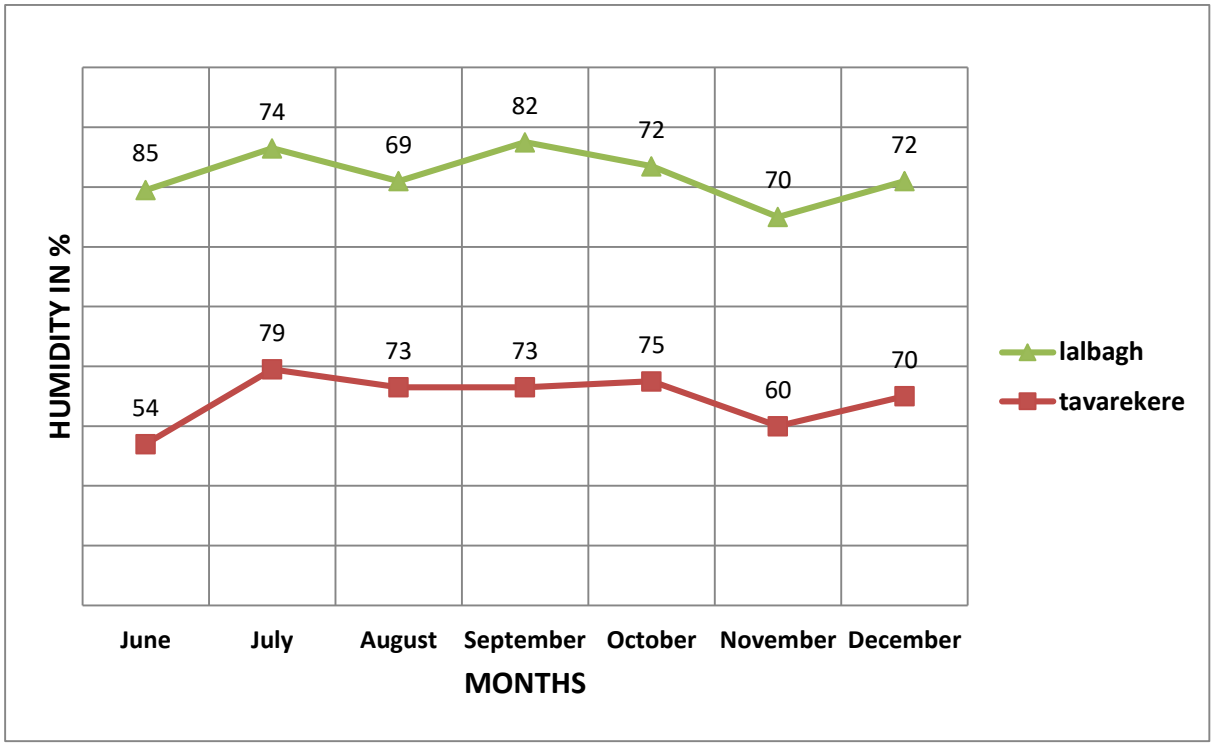

Fig 4: Distribution of Humidity at the Study Area

Table 3: Diversity Indices of Spiders:

	Lalbagh	**Tavarekere**
Taxa_S	11	17
Individuals	50	79
Dominance_D	0.1224	0.1639
Simpson_1-D	0.8776	0.8361
Shannon_H	2.215	2.239
Evenness_e^H/S	0.8331	0.552
Margalef	2.556	3.662

V. CONCLUSION

The present study is done at two urban areas to check their diversity and also gives a background for any future studies being done this department. The survey was conducted for a period of seven months from June to December 2019. They help in maintaining the ecological balance of nature as they feed on large population of insect preys. It was seen that Tavarekere Park showed good species diversity and richness as compared to Lalbagh Botanical Garden in Bangalore South as the diversity indices is 2.239 and 2.215 respectively and also with richness being 3.662 and 2.556 respectively. This helps in showing that they are both good predators and preys. There is a need to study the seasonal variation of the spider fauna in these regions and the conservation of this ecosystem which has been a habitat for multiply species of spider fauna.

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Conservation of Italian Autochthonous Domestic Pigeon Breeds

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***Abstract*—** *In this work it is proposed an analysis of the conservation status of Italian autochthonous domestic pigeon breeds. As like as other domestic species, the domestic pigeon is undergoing a rapid decline. In Italy the pigeon breeding is an ancient practice anterior to the Roman period. Actually we have 11 autochthonous breeds, here described in their mainly characteristics, and for everyone is proposed the population size and the perspective of conservation for the future. An important and fundamental impulse to the conservation of these breeds has been given by the Italian Pigeon Breeders Federation – FIAC and its numerous members, which have preserved the biodiversity heritage of the domestic pigeon in Italy. Of the Italian autochthonous breeds, only three are considered not at risk, while the other are in the range size for the breeds considered threatened. Two breeds, the Rondone frill and the Parma Occhialone pigeon are now present with numbers that classify them in the "critical" category. For the Rondone frill a numerical increase program is being developed, for the Parma Occhialone pigeon the FIAC has activated a recovery and diffusion program.*

To guarantee a future for biodiversity of this interesting domestic species in Italy, will be necessary to encourage the breeding of these Italian autochthonous domestic pigeons.

Keywords*— *pigeon breeds, conservation status, domestic pigeon, Italy, population size.

I. INTRODUCTION

It has been estimated that since domestication, in the last 12,000 years, over 7616 breeds have been selected by 30 species of domestic animals. In the past hundred years, around 22% of known animal breeds have become extinct and another 27% have varying degrees of risk. It is also reported that approximately two breeds of poultry and livestock are lost every week (Grunenfelder, 2001; Rischkowsky & Pilling, 2007; Bigi & Zanon, 2008).

The autochthonous domestic breeds have been an integral part of the livelihoods and traditions of different communities over the years and the loss of one defined race is a loss of the cultural identity and heritage of that community (Belew *et al*., 2016). Losing these breeds is like losing a comprehensive insurance policy against future food security threats (Shah *et al*., 2016). The indiscriminate crossing between exotic breeds and indigenous animals was judged to be the main cause of the losses, as well as the risk for the existing breeds. It may be too late for many livestock and poultry breeds in Europe; also in developing countries the loss of diversity and indigenous animals is present, even if it is less high. The continued import of highly productive animals from developed countries is believed to be the most significant threat to domestic animal diversity in developing countries (Hanotte & Jianlin 2006).

In this context fits the domestic pigeon, which, like other domesticated species, is undergoing a rapid decline. In this work, the native domestic breeds of pigeons in Italy are described; the Author wants to define their *status* and perspective for future protection from extinction.

II. THE BREEDING OF PIGEONS IN ITALY

The domestic pigeon is a direct descendant of the Rock Pigeon *Columba livia* Gmelin 1789, a wild species native to Europe, North Africa, the Middle East, and South Asia. The species was probably domesticated at several times and places. Archeological evidence points to human use of pigeons as a food source as early as the Pleistocene (~ 10,000 years ago) in the Fertile Crescent, although whether this entailed domestication is not known. Ancient Egyptians began using pigeons for ceremonial and culinary purposes at least 4000 years ago, and later as harbingers of the progress of the Nile's annual flood. Ancestors of some modern breeds were probably developed between 2000 and 860 years ago. The geographic centers of biodiversity for pigeon appear to be the Middle East and South Asia (modern India and Pakistan); from these geographical areas the domestic pigeons were then spread all over the world (Giusti, 1996).

In Europe, the popularity of the pigeon breeding raised markedly in the 17th Century. With the publication of the works of Charles Darwin "*The origin of the species through natural selection*" and "*The variations of plants and animals in the domestic state*", which took place around in the mid-1800s, we can point at the born of the modern selective pigeon breeding. The theses supported by Darwin found confirmation of their validity and were rapidly implemented in pigeon breeding, leading to the creation of numerous new varieties. The working material was supplied, as well as by the breeds already existing in Europe, by new breeds that were imported from the eastern colonies especially in England-earth, then the center of a vast empire. (Pizzuti Piccoli, 2016)

In all Europe about 1050 of pigeon breeds are present today; of these many are endangered (AA.VV., 2018).

In Italy probably the breeding and use of the pigeon was introduced by the Greeks to Magna Grecia. From Sicily the interest in breeding spread rapidly to Rome and Italy, especially in the area around Modena which constituted an important Roman colony for its geographical position. Varrone Latino (1st century B.C.) in his works deals with details of the breeding of pigeons, reporting that in his day a couple used to be sold for 1,000 sesterces and that there were pigeon lofts with 5,000 animals. Columella (1st century A.D.) teaches how to build a pigeon loft how to breed pigeons. (Calzecchi Onesti, 1977; Traglia A., 1996).

The historian Plinio in his "*Historia naturalis*," as well as Frontino, tell that in the year 43 B.C., when Modena was besieged by Marco Antonio, the connections between Decio Bruto, besieged in the city, and the encampment of Consul Irzio were possible thanks to messengers pigeons. (Malossini, 2011)

The Italian Ulisse Aldrovandi, from Bologna, was the first in Europe to write, around 1600, a scientific treatise on the breeds of pigeons existing in his time.

In Italy, in the city of Modena, the "game of flying" of the Triganini pigeons had been widespread since time immemorial. Many testimonies have been handed down about it, the most famous of which is that, dating back to 1614, by Alessandro Tassoni who, in his heroic poem "La secchia rapita", speaks of the people dedicated to this sport, that were called "*triganieri*" (Puliatti, 1989)

The period of greatest diffusion of this custom was at the beginning of the 1800s, when many people, belonging to every social class, spent most of the day on the roofs, taken as they were from the passion for this game. (Polacci, 1978)

Today, Italian pigeon breeders are organized in many local associations and, at a national level, in a Federation, the Italian Federation of Pigeon Breeders (acronym FIAC).

The F.I.A.C. was founded in 1977 and today brings together 27 Italian Associations of pigeon breeders and 12 Groups of breeders specialized on one or more particular breed. Every year the FIAC organizes the National Pigeons Exposition, a national show where it is possible to admire the best specimens bred in Italian lofts.

Despite this passion, we live the great problem of the conservation of biodiversity of domestic pigeon breeds.

In recent decades one of the greatest problems we face is the conservation of all breeds of domestic pigeons, which risk disappearing forever. Some breeds have already disappeared in Europe and others are reduced to a minimum; all these domestic varieties will live as long as there are fans that will breed them with many sacrifices and a lot of passion. (Pizzuti Piccoli, 2011)

III. ITALIAN AUTOCHTHONOUS BREEDS

Today in Italy 10 pigeon breeds are officially recognized as Italian breeds. For each breed, the FIAC has, from years, established a standard with breed characteristics.

The breed standard is the more possible accurate description of physical and behavioral characteristics that an "ideal" specimen of a given animal breed should possess; therefore it serves as a guide for breeders to determine which distinctive features to privilege in the selection.

In the Standard the breed is described in its general aspect and in the details of the individual anatomical parts. The possible variants are listed (for example the various colors of the plumage), any points of particular value in the specimen that should be enhanced for the reproductive selection are listed. The Standard also report the inadmissible defects identified, which lead (or should lead) to the exclusion from reproduction, in order to maintain the morphological homogeneity of the breed and exclude unwanted mutations that can lead, for example, to health problems.

A French breed, the Roman pigeon, is considered to be a descendant of an Italian breed perfected in France.

In addition to the official breeds, of which the FIAC is the custodian of the standards and, above all, has guaranteed conservation up to the present day, there are many local breeds that have disappeared over time; is this the case of the Parma Occhialone pigeon, first considered extinct and then "rediscovered" in Sicily, treated separately below.

The Piacentino pigeon

The first evidence of Piacentino pigeon (Fig.1) dates back to the nineteenth century in Emilia Romagna, precisely in the neighboring countryside of Piacenza, it probably derives by crossing large indigenous pigeons, Roman and Bagdad pigeons. It is a large-sized pigeon, of robust constitution with a wide chest and horizontal position. The minimum weight for adults varies from 850 grams for females to 900 grams for males. The length from the tip of the beak to the end of the tail varies from 34 to 36 centimeters, while its height varies from 31 to 33 centimeters. Piacentino pigeons have a large, smooth, convex and well rounded head in all its set; it must form a continuous and rounded line that starts from the tip of the beak to arrive harmoniously at the junction of the neck with the neck. It has large eyes with dark iris in the white coloration (vetch eye), orange red (rooster eye) in all the other colorations. The rings of the eyes appear broad, not excessively protruding, nor too fleshy with a bright red color (cardinal red). Medium length beak often joins to the forehead without restriction (no stapling). The beak is pink in color in the white pigeons and more or less light horn in the remaining colorations. The wattles are white, wide and well extended, but smooth and not too pronounced; they must not break the continuous line of the profile between the forehead and the beak. Well carved throat with no hint of dewlap. Strong, medium-length neck, wide at the attachment with the chest, it reduces harmoniously up to the throat. It has wide, prominent, rounded chest, deep but not visible sternum. Wide back, medium length carried horizontally. It has large, tightly fitting wing shields that cover the back. Feathers well closed, lying above the tail and slightly shorter than it. Tail of medium length, compact, slightly raised, it goes beyond the end of the flight feathers by 2 or 3 centimeters. Sturdy, medium-length legs, well spaced from each other, with slightly angled profile. It has thighs clearly visible, fingers with nails of the same color as the beak (AA.VV., 1999).

The Romagnol pigeon

Romagnol (Fig.2) was already selected in the second half of the 19th century, starting from big pigeons bred for centuries in the farms and colonies of the provinces of Romagna and Bolognese. The name Romagnol is attributed to this pigeon by prof. Alessandro Ghigi in 1898, given its diffusion in the districts of Romagna. Romagnol is a big size pigeon, with prominent and well rounded wide chest. It has a horizontal standing and an average high position on the legs that are characterized by the presence of well-developed spurs and slippers. The minimum weight in adults is 800 grams for females and 900 for males. The pigeon has a height from 31 to 32 centimeters and his length (from the tip of the beak to the end of the tail) is from 32 to 34 cm.

The head is smooth and well rounded without interruptions or flattening, it has a rounded forehead that forms with the beak an obtuse angle. The beak is of medium length rather carried almost horizontal. The wattles are smooth, fine and white in color. Well carved throat with no hint of dewlap. It has a wide back carried horizontal that narrows towards the tail. Strong wings with broad shoulders adhere to the body and cover the back and lie on the tail without crossing each other. The plumage is smooth, shiny, thick, well adherent to the body with large but not soft feathers (AA.VV., 1999; Rubboli & Mazzanti, 2013).

The Florentine pigeon

The Florentine is an Italian breed of ornamental pigeons very ancient, but little known (Fig.3); it is very difficult to trace its origin and follow its diffusion in Europe. Already mentioned as a breed in the 1700s, the ancestor of Florentine is a robust pigeon, used for the production of meat, very characteristic for its short and arched shape. N the modern specimen the head is robust, the neck long, the tail is carried very vertical and divided into two equal halves, the legs are long and robust but the foot is small compared to the overall appearance of the pigeon. The crossing probably with Triganino Modena leads to the current design of the color pattern which is called "gazzo" (an Italian word that remember the magpie and his color pattern), the background color is white, the wing shields are colored, the tail including the wedge and the rump, the head with a mask that starts from the nape of the neck and down 3-4 cm below the beak. In twenty century we approach the current form that is, a robust pigeon, long and straight neck, short and vertical tail. It was widespread in northern Italy, especially in the Alpine area, from this area spread in Austria. The Florentine is bred also in Germany. The most common colors are red, black, brown, blue with or without black rods and yellow (AA.VV., 1999)

The Rondone frill

The Rondone frill is a very old breed, whose existence is already testified at the beginning of the 19th century (Fig.4); created in the city of Reggio Emilia and surroundings, in all probability through crossings between the Italian Owl and the Damascene pigeon.

There are only two colorations of Rondone frill, the ice with black bars ("*lattato*" in Italian) and the ice with

hammered design ("*fagiano*" in Italian), in both these varieties the background color must be as clear as possible and the neck must not have any grayish or rust tinge. The Rondone frill is a medium-sized pigeon, slightly low on the legs, slender in shape with an inclined standing downwards. The head has a long shape, the neck is full and the upper cranial part is slightly flattened, the forehead is very wide and must form a continuous line with the beak. The eye of the Rondone frill is orange in color while the eyelid and the ocular ring must be black / blue. The beak, black in color, is robust and short, just below the beak there is a well-developed dewlap, in the center of the chest there is an evident frill. The wide and rounded chest is carried slightly raised, while the back and tail are gradually inclined downwards and the tail must never touch the ground (AA.VV., 1999).

The Italian owl

The Italian owl is an ancient breed created in the city of Reggio Emilia and bred there for centuries (Fig.5); from its homeland of origin this pigeon also derives the name with which it is commonly defined "Reggianino". Its origin can be placed at least around the 16th century. According to some authors, this breed was born from the crossing of neck frill pigeons, of African origin, with local pigeons, perhaps of the Triganino Modena type.

It is a short, taller than long, with small neck frill, weighing about 280/300 grams. Higher on the tarsi than the other breeds of ties, it has a tendency to be on the tip of the toes, horizontal bearing and cubic and angular head. The head must be short and wide, angular, seen in profile and from above it is square in shape since it is flattened in its upper part. The cranial vault tends to be parallel to the midline of the beak. The eyes are bulging, large and bright; located approximately 3/5 forward from the back of the skull, more or less intense orange iris, dark (vetch eye) in white subjects; in the piebald the irises can be both orange and dark but both of the same color. The beak is short, wide at the insertion with the skull, long as wide, straight, it detaches from the forehead forming with it a well marked angle. The throat must have a pronounced dewlap. The plumage is tight-fitting, well adherent to the body, compact. The breed has several well-defined and pure colors (AA.VV., 1999).

The Ascolano pigeon

The Ascolano pigeon (Fig.6) is the reconstruction of a very ancient breed, of which the first information is found in a 1768 paper; in the painting of 1486, "Annunciation with Sant'Emidio" by the famous painter Carlo Crivelli, it is represented an ancestor of the modern Ascolano pigeon.(Cantalamessa, 2008).

Its breeding was once widespread not only in the Marche (Central Italy), but also in Emilia-Romagna and Campania. This breed is probably the ancestor of Romagnol.

The Ascolano pigeon is a pigeon with a large conformation, with a wide chest, medium-length body with an inclined bearing downwards, forming a line with the tail, which touches the ground at the tip. Small head in proportion to the structure of the body, smooth and rounded, with long, thin and straight beak. It has an abundant plumage of the body, made up of wide, soft and slightly loose feathers. Sturdy wings and wide wing shield, carried not too close to the body and laid down over the tail without crossing. The legs are medium length, strong and well placed with heavily feathered tarsi and fingers with medium length feathers. The colorations recognized by the standard are black, white, brindle, butterfly and black piebald. The black color must be intense and uniform with the neck rich in beetle green reflections, in the other coats it must have white feathers distributed uniformly on a dark background, and in the piebald the parts of the two colors must have equivalent extensions (AA.VV., 1999).

The Bergamasco pigeon

Breed created in the countryside of Bergamo in the nineteenth century, probably through crosses between indigenous and Polish pigeons of the English type, in order to obtain subjects that corresponded to the needs of food production for men (Fig.7). This pigeon was widespread in the Bergamo's country land mainly for food use. Its origins are not exactly known, it was mentioned without particular descriptions in the "Picturesque dictionary of natural history and manufactures" by Guèrin in 1845; however, since in the early 1950s, it was present a considerable number of specimens very similar to the modern pigeon.

The Bergamasco pigeon has a very developed red colored eye rings, The back of the head is adorned with a shell tuft, with wide and well developed rosettes, placed low and adherent to the nape and neck (looking at the pigeon in front, both the tuft and the rosettes must not be visible) very thick mane, slanted and long back, thin neck, head with broad forehead, detachment clearly visible between the forehead and the beak, shoulders marked and well delineated from the chest which, although wide, is not prominent. The legs are strong, with a slightly angled profile; bright red tarsi with long fingers. The Bergamasco pigeon has the colors white, black, brown, red, yellow, almond. (AA.VV., 1999; Comi, 2011)

The Triganino Modena pigeon

Ancient breed of pigeons, whose origin is supposed to date back to the fifteenth century, the Triganino Modena pigeon (Fig.8) have been bred for many centuries in the city of Modena (Emilia Romagna). Here, until the early 1900s, it was used as a messenger over small and medium distances, but, above all, it was used for a characteristic flight game. It is not possible to establish precisely what its progenitors were but, in all likelihood, it can be said that Triganino was selected through crosses between indigenous pigeons and pigeons of eastern origin, of the type of the current "hen pigeons"; this would also explain the characteristic shape of the Triganino. The name "Triganino" would derive to this pigeon from the speed and grace of its forms that make it resemble a dove (*τρυγόνι* in ancient Greek). The Triganino Modena pigeon is a pigeon with an elegant shape and a lively temperament; it has a reduced size, is short, rounded, slender on the high tarsi, well balanced between head and tail; it moves with promptness and agility, has rapid and iridescent flight. Its total length, from the chest to tip of the tail, is about 23 cm. The breed is divided into two large groups of colors: "*Schietto*" and "*Gazzo*". The term *Schietto* derives from the word "*sciét*", in the Modenese dialect, that means unmixed, pure, in reference to the color of the body, which is totally colored. The *Gazzo* is so called because of the similarity of its design with that of the Magpie *Pica pica* (Linnaeus, 1758), "*gazza*" in Italian. The Gazzo has a white body, with pigmented head, wings, tail and undertail. The dividing line of the colored part, on the nape, should reach the height of the lower contour of the eye. The Triganino Modena pigeon, among all the breeds of pigeons, is certainly the one that includes the most colors; of the Schietto and Gazzo varieties there are over 100 official colors (Polacci, 1978; AA.VV., 1999; Vaccari & Zambon, 2014).

The Italian beauty Homer

The breed (Fig.9) was selected from 1980, starting from racing pigeons of above average size, with very developed wattle. It is very similar to a robust muscular, compact race pigeon, with an average erect bearing; it has a rounded head, characterized by well-developed wattles, conical in shape, which increase in volume with the progress of the subject's age. The head appears robust, proportionate to the size of the pigeon, with a gently rounded profile, without interruptions or stapling, from the forehead to the nape it slightly widens towards the rear and continues in the full neck.

The eyes have an iris of a color ranging from bright red to orange, the eye contour is formed by two regular concentric circles, light in color and moderately developed. The beak is strong, well closed, formed by two parts of equal strength, slightly obtuse at the end; the continuation of the beak commissural line must pass through the center of the eye. This breed has well-developed wattles, both seen from the side and from above, they have a triangular shape, they begin shortly after the tip of the beak, gradually rising and widening towards the forehead and ending clearly, detached and higher than the curve of the forehead. In young subjects they have a fine, regular texture and are divided by a longitudinal furrow; in adults they gradually increase in volume, but without forming irregular growths in the two parts of the beak, maintaining their compactness and regular shape. In adults, a light wattle is allowed on the lower part of the beak.

The Italian beauty Homer has various colors and plumage well adheres to the body **(**AA.VV., 1999).

The Sottobanca pigeon

The study carried out on the origins of the Sottobanca pigeon traces its creation to the second half of the 1500s in the Modena countryside (Fig.10). The naturalist Ulisse Aldrovandi (1522 - 1605) had several sketches drawn by the painter Jacopo Ligozzi (1547 - 1627) depicting the pigeons raised in the second half of the 1500s, one of which can be traced back to Sottobanca. The name "*Sottobanca*" seems to derive from the custom of these birds to nest and incubate eggs on the ground, under the tables in the arcades of the rural farmhouses where, in the Modena dialect, the table is called "*banca*". We can see in this depiction that the Sottobanca had a more or less shell-shaped tuft, a relatively short and thick beak compared to all the other pigeons of the time and the shape defined as a boat. Today we can therefore say that the ancestors of the Sottobanca were, already in that time, pigeons that had characteristics very similar to the current Sottobanca, characteristics that have remained unchanged over time. As with all the other indigenous breeds of poultry, the Sottobanca has also undergone the evolution of the times and the transformation of the rural world; today his breeding is almost exclusively aimed at the production of subjects intended for exhibitions, with good typicality and morphologically corresponding to the Breed Standard. This modern kind of selection often penalizes the breeding capacity of specimens.

The Sottobanca is a pigeon rather high on the legs, with proud bearing, horizontal body and slightly raised tail. The head is robust, slightly flattened at the top, with a broad

and moderately ascending forehead. The back of the head, the nape of the neck, is adorned with a thick shell-shaped tuft. The beak is moderately short, robust, rounded and slightly curved at the tip. The chest is large, prominent, rounded while the back is wide, short and robust, carried as horizontal as possible. (AA.VV., 1999; Garagnani, 2015)

The conservation of the Parma Occhialone pigeon.

The Parma Occhialone pigeon, an ancient Italian breed, has a history in its own (Fig.11). Today the FIAC, with the involvement of some pigeon breeders, is trying to save this pigeon from extinction. Considered extinct around the mid-2000s, in reality the breed has always been bred, albeit with few specimens, by breeders in the Emilia Romagna area.

Particular impetus for the conservation of the breed was given by Mr. Antonino Palazzolo, of Palermo (Sicily), that years ago bought some specimen from a breeder from the northern of Italy and who has preserved the original genetic heritage of this breed over the years.

There are many historical sources that describe this pigeon as early as 1800. In the book "*domestic pigeons and breeding*" by Alessandro Chigi of 1950, it is reported that the Duchess Maria Amalia of Austria had introduced in Parma, towards the end of the 1700s, many wattle pigeons from Vienna, from which Parma Occhialone pigeons were derived, through crossings with the local Italian Owl and Belgian racing pigeon (Giachetti, 1914; Chigi, 1950).

The breed is still very common in the 70s in Emilia Romagna, so much so that the prestigious specialized magazine "*Colombicoltura*" dedicates a monograph to the Parma Occhialone pigeon in 1977 (Morini, 1977). The presence of this breed is also evidenced by the participation in national pigeon expositions; until the early eighties the breed was regularly exposed even if with very low numbers, for example in 1979 only 10 specimen of Parma Occhialone pigeon were expose in the national pigeon exposition of Parma (Casadei, 2003).

It is therefore clear that the breed, not being completely extinct, has however continued to persist in many places in northern Italy.

Thanks to the work of Mr. Palazzolo, today the FIAC has created a task force to preserve the genetically characteristics of this breed and spread its breeding.

The Parma Occhialone pigeon is classified in the "barb pigeon" group. Morphologically is a medium - small pigeon, with a slightly inclined posture towards the rear, with a good attitude to flight. It weighs around 400 grams; the tail is about 1,5 cm longer than the wings.

The head is rather large, high and broad forehead, which joins the beak forming a continuous line; it has a well rounded head, with or without tuft. The beak is on average length, very robust and large, rounded on the tip, flesh-colored. Smooth nasal wattle in the young and in the females, are more developed in the males and in particular in the adult males, of pink color powdered with white. The eyelids are very large and developed, about 6 mm wide, forming a showy circle (consisting of two three rows of finely granulated rings) of red color. The pigeon has a fairly long, stocky neck with a well-rounded throat. The chest is rounded, not too wide, without a tie. The back is carried sloping and forms a continuous line from the shoulders to the tail. The pigeon has no plumage on legs and plumage well adherent to the body; it is bred in the colors yellow, red, black and white, piebald in all colors (Mc Neillie, 1981).

IV. STATUS AND STRATEGIES FOR CONSERVATION

For the Italian indigenous breeds, we report below the population estimate obtained from the data collected by breeders registered in the Italian Federation of Pigeons Breeders (Table 1). In the calculation are indicated the pigeons bred by official breeders, a 30% increase has been added to this number, evaluating an increase due to the percentage of breeders who are not included in FIAC official members. In the case of Triganino Modena pigeon, Italian owl and Florentine, in assessing the population estimate, references was also made to the number of couples raised in other European countries (especially France, Germany and Austria) where these breeds are widespread. The numerical estimate, although not precise for the difficulties of finding information, still allows us to identify the order of magnitude of the existing populations, and to evaluate their conservation status.

For the determination of breeds at risk, reference was made to the criteria reported in "The Global Databank" published by FAO, which classifies domestic breeds into seven categories: extinct, critical, threatened, not at risk, unknown, sustained criticism, sustained threatened (Sherf, 2000).

The categorization is based on the overall size of the population, the number of breeding females and the trend of the size of the population, i.e. whether the population size is increasing, decreasing or stable. If the categorization of a particular breed is at its limit, a further consideration is in the categorization is whether conservation is active and programs for critical or endangered populations are in place.

The general guidelines used to determine the risk status are as follows; however, the guidelines have been adapted to the biology of the species, which is monogamous (we report the pairs for each breed). In the Table 1 it is also reported the actual trend (if decreasing, stable or growing) for the breeds.

Extinct

A breed is classified as extinct if the breed population can no longer be easily recreated. This situation remains irreversible and becomes absolute when there are neither breeding males nor breeding females. In reality, extinction can be declared well before the loss of the last animal, gamete or embryo.

Critical

A breed is classified as critical if the total number of breeding females is less than 100; and therefore, in the case of the pigeons, if the pairs are less than or equal to 100, being the pigeon a monogamous species.

Threatened

A breed is classified as endangered (threatened) if the total number of breeding females (or couples for the pigeon) is between 100 and 1000.

Not at risk

A breed is classified as "not at risk" if the total number of breeding pairs is greater than 1000 or if it approaches 1000 and is increasing.

Unknown

It is a category reserved for breeds of which numerical data relating to the individuals are not known. In this category we could find all the previous categories, but we are not able to define their status.

Domestic breeds can be further classified as critical - supported and threatened - supported; these categories identify populations with critical or endangered status for which conservation programs are active (both managed by private and public companies).

Table 1. Population size and status for Italian authocthonous breeds.

Breed	**Population estimate (expressed in pairs)**	**Status**	**Trend**
Ascolano pigeon	200	Threatened	Stable
Bergamasco	350	Threatened	Stable
Italian Owl	> 1000	Not at risk	Growing
Rondone frill	80	Critical - supported	Growing
Florentine	650	Threatened	Stable
Parma Occhialone pigeon	55	Critical - supported	Growing
Piacentino	325	Threatened	Decreasing
Romagnol	800	Threatened	Decreasing
Sottobanca	120	Threatened	Decreasing
Triganino Modena	> 1000	Not at risk	Growing
Italian beauty Homer	> 1000	Not at risk	Growing

V. CONCLUSION

Of the Italian autochthonous breeds, the Italian Owl, the Triganino Modena and the Italian beauty Homer are certainly three out of risk breeds without problems for their future conservation. The Rondone frill and the Parma Occhialone pigeon are now present with numbers that classify them in the "critical" category. For the Rondone frill a numerical increase program is being developed, for the Parma Occhialone pigeon the FIAC has activated a recovery and diffusion program.

For the three typical breeds of Emilia Romagna, Sottobanca, Piacentino and Romagnol we have defined a threatened status; their situation is worrying because of the decreasing trend.

Also for the breeds of Ascolano pigeon, Bergamasco and Florentine there is a worrying situation because their populations are represented by a few hundred couples (threatened category) and their populations are stable and not growing.

In conclusion, it seems clear to the Author that the Italian Pigeon Breeders Federation – FIAC and its numerous

breeders have preserved the biodiversity heritage of the domestic pigeon in Italy. Today the conservation of these breeds is certainly more difficult for many factors, especially in the average age of breeders, around 60 years, and the lack of new and young breeders.

Raising pigeons today is very difficult in Italy, because of the current health regulations that impose many restrictions.

The work started and continued by the Italian pigeon breeders will probably have to be encouraged in order to lead, in the coming years, to a numerical increase in all breeds, to guarantee a future for domestic biodiversity of this interesting species.

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Fig.1: The Piacentino pigeon (Photo by R. Comi – FIAC)

Fig.2: The Romagnol pigeon (Photo by R. Comi – FIAC)

Fig.3: The Florentine pigeon (Photo by R. Comi – FIAC)

Fig.4: The Rondone frill (Photo by R. Comi – FIAC)

Fig.5. The Italian owl (Photo by R. Comi – FIAC)

Fig.6: The Ascolano pigeon (Photo by R. Comi – FIAC)

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Fig.7: The Bergamasco pigeon (Photo by R. Comi – FIAC)

Fig.8: The Triganino Modena pigeon (Photo by R. Comi – FIAC)

Fig.9: The Italian beauty Homer (Photo by R. Comi – FIAC)

Fig.10: The Sottobanca pigeon (Photo by R. Comi – FIAC)

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Fig.11: The Parma Occhialone pigeon (Imagine by Mc Neillie A., 1981)

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Potato Skin: A Potential Biostimulating agent for used Motor Oil Biodegraders

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***Abstract*— *The potential of potato skin (PS) to enhance bioremediation of soil polluted with used motor oil was investigated gravimetrically for a period of 42 days. Polluted soil was amended with 5%, 10% and 15% (w/w) of PS. Loss of total petroleum hydrocarbon (TPH), microbial growth and germination indices were all investigated throughout the study period. At the end of 42 days, there was significant oil loss of 73.85% in the amended soil. Hydrocarbon-utilizing bacterial (HUB) counts were significantly higher($P \leq 0.05$) in the amended option ranging from 6.7 x 10^6 to 22.3 x 10^6CFU/g. The HUB isolated from the oil-contaminated soil were identified tentatively as belonging to the genera: Bacillus, Arthrobacter, Rhodococcus, Corynebacterium, Pseudomonas, Staphylococcus and Acinetobacter. Similarly, fungal counts ranged from 4.8 x 10^5 to 59.0 x 10^5 CFU/g. Aerobic fungi isolated were identified tentatively as Aspergillus niger, Aspergillus sp., Pennicillum sp., Phialophora sp., Cladosporium sp. and Verticillum sp. Germination index of 69.46% was recorded in the amended option. Oil loss and microbial growth were significantly higher ($P \leq 0.05$) in the amended option than the control option. Potato skin, therefore can offer a good alternative in bioremediation of soil polluted with used motor oil.*

***Keywords*— Bacteria, fungi, potato skin, bioremediation, used motor oil.**

I. INTRODUCTION

Motor oil is a complex combination of hydrocarbons with other organic compounds, including certain organometallic compounds, it is used to lubricate the components of an automotive engine, to keep the engine and its whole operation working smoothly. Used motor oil (UMO) also contains impurities such as heavy metals, as well as polychlorinated bi-phenyls [1]. Used motor oil also contains toxins and mutagenic polycyclic aromatic hydrocarbons (PAHs), which accumulate gradually with miles due to direct fuel leakage into the motor oil, as well as the build-up of incomplete combustion products [2].

There have been growing use of motor oil due to the presence of different types of vehicles and machinery. Sadly, soil pollution is growing rapidly with used motor oil due to global growth in the use of petroleum products [3]. Spilling the used motor oils involves hydrocarbon damage to our natural environment with hydrocarbons [4]. Contaminants have been identified as being capable of accumulating and toxic to biological systems (plants and animals) [1].

A variety of revolutionary physical and chemical techniques are available for the remediation of hydrocarbon-contaminated areas, such as soil washing, vapor extraction, encapsulation and solidification/stabilization [5]. These approaches, however, are expensive and can be only partially effective. Furthermore, the field utilization of these intense techniques can be limited by public pressures [5].

It has been widely demonstrated that microorganisms possess inherent abilities to degrade hydrocarbons and UMO is not an exception. The degrading organisms utilize hydrocarbons as carbon source. It has been reported that while hydrocarbons are excellent carbon sources for organisms, they are incomplete foods in that they contain insufficient quantities of other nutrients such as nitrogen and phosphorus required for

microbial growth and activities [6]. Lack of essential nutrients, such as nitrogen and phosphorus, is one of the major factors affecting biodegradation of hydrocarbon by microorganisms in soil. Therefore, the addition of amendments (biostimulation), such as biochar, ash, pig manure, sewage sludge, is effective in lowering the metal and PAHs toxicity of soil and provides a slow release of nutrient sources such as N, P, K to enhance the bioremediation process [7]. The nitrogen amendment on microbial activity and/or petroleum hydrocarbon degradation has been widely demonstrated [8,9]. Organic wastes vary in their content of nitrogen and/or phosphorus and this reflects the extent they perform in their biostimulatory activities. This work was therefore carried out to assay for the biostimulatory potential of potato skin as an alternative biostimulation candidate for UMO-impacted soil based on its profile of nitrogen and phosphorus content.

II. MATERIALS AND METHODS

Collection of Samples

The soil sample was collected from the Faculty of Agriculture farm, University of Nigeria, Nsukka, Southeast Nigeria in sterile polythene bags at a depth of 0-15 cm from different sampling sites and transported to the laboratory for analysis. Used motor oil was collected from the Mechanic village, Nsukka, Enugu state, Nigeria. Potatoes were bought from Ogige Market, Nsukka and peeled to take their skin. Seeds of cucumber (*Cucumis sativus*) were obtained from the Department of Crop Science, University of Nigeria, Nsukka.

Determination of Physicochemical Properties of Soil and Potato Skin

Physicochemical properties of soil and potato skin such as particle size distribution, percentage moisture content, pH, total organic carbon (%TOC), % nitrogen content and total phosphorus were analyzed following standard protocol [10]

Soil Preparation for Bioremediation

A 1kg quantity of soil (sieved with 2 mm mesh size) was placed in sterile polythene bags and polluted with 10% (v/w) UMO, and left undisturbed for 48 hours. The polluted soil was amended with air-dried and pulverized potato skin at the concentrations of 5%, 10% and 15% w/w. Soil neither polluted nor amended served as the positive control while polluted soil without amendment served as the negative control. The moisture content of the soil was adjusted to 60% water holding capacity by the addition of sterile distilled water (50 ml, three times weekly) and the set-up incubated at room temperature (28 ± 2°C). Periodic triplicate sampling from each set-up was carried out at 7-day intervals for isolation and enumeration of microorganisms (fungi and bacteria) and determination of residual UMO.

Determination of Extraction Efficiency of Different Solvents for Diesel Oil

The extraction efficiency of three organic solvents, namely: dichloromethane, diethylether and n-hexane for used motor oil was predetermined in order to examine the rate at which the solvents would be able to extract the UMO pollutant from polluted soil. Extraction efficiency study was carried out gravimetrically. Briefly, a 20 g portion of soil mixed with 2 mL used motor oil was homogenized and left for two hours in a 250 mL flask. Thereafter, the soil-oil mixture was mixed with 80 mL of the different solvents and the set-up shaken for eight hours at 180 rpm. The solution was then filtered using a Whatman No 4 filter paper and the weight of the extracted oil recorded. The extraction efficiency of the organic solvents for UMO was then determined by weight difference following the formula [11]. The experiment was carried out in triplicates.

$$\text{Extraction efficiency} = \frac{\text{Weight of 2 mL UMO} - \text{Weight of oil extracted from soil}}{\text{Weight of 2 mL diesel oil}} \times 100$$

Soil Preparation for Bioremediation Study

A 1 kg quantity of the sieved soil was placed in sterile polythene bags and 10 % (v/w) of UMO was added, mixed thoroughly, and left undisturbed for 48 hours. After two days, 5%, 10% and 15% (w/w) pulverized potato skin were respectively, introduced into the UMO-polluted soils and mixed thoroughly. Soil sample contaminated with 10% (v/w) UMO without amendment served as control. The moisture content of the soil was adjusted to 60% water holding capacity by the addition 50 mL of sterile distilled water (three times weekly) and the set-up kept at room temperature (28±2°C). The experiment was set up in triplicates.

Determination of Percentage Bioremediation

Periodic sampling from each polythene bag was carried out every seven days in order to determine residual UMO. Gravimetric method [12] was modified slightly and employed in the determination of UMO present in both the unamended control soil and all the amended microcosms. Composite polluted soil samples weighing 5 g were put in a 50 mL flask and 10 mL of diethyl ether was added. Diethylether was used

because it gave the highest extraction efficiency (see result section). The set-ups were shaken with a rotary shaker at 180 rpm for two hours to allow for an efficient and complete oil extraction with diethyl ether. The mixture was then filtered with a whatman No 4 filter paper. The filtration was done repeatedly two times to ensure complete extraction of the liquid phase. The filtrate was diluted by adding 50 mL of diethylether to 1 mL of the extracted UMO and the absorbance of the solution measured at 460 nm (Shimadzu UV 1800) using diethylether as blank. The total petroleum hydrocarbon (TPH) was estimated by extrapolating from a standard curve derived from different concentrations of fresh UMO diluted with diethylether. Percent remediation (R) was calculated using the following formula:

$$R = \frac{TPHi - TPHr}{TPH} \times 100$$

Where TPHr and TPHi are residual and initial TPH concentrations

Enumeration and Identification of Microorganisms

The indigenous hydrocarbon-degrading flora was enumerated following standard bacteriological and mycological methods. For bacteria, 10-fold serial dilutions were made from oil-polluted soils undergoing treatment and 0.1mL aliquot of the appropriate dilutions were spread on nutrient agar plates. Triplicate plates were incubated at 30 °C for 24 h before the bacterial colonies were counted. Hydrocarbon utilizing bacteria (HUB) in the soil samples were enumerated using modified mineral salts medium [13]: 1.8 g K_2HPO_4, 0.1 g $CaCl_2$, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar, in 1000 ml distilled water, pH 7.4, using the vapour phase transfer method [14]: briefly, a filter paper saturated with sterile UMO was aseptically placed on the inside of the inverted petri dishes of the inoculated mineral salt agar and the culture plates were incubated at 28 °C for 7 days [15]. Morphologically distinct hydrocarbon-utilizing bacteria (HUB) were randomly picked and pure isolates were obtained by repeated sub-culturing on nutrient agar. The bacterial isolates were identified tentatively by Gram reaction and biochemical characteristics.

For fungal enumeration and identification, ten-fold serial dilutions were made by suspending 10 g of treated soil in 90 ml of sterile distilled water. The soil suspension was shaken vigorously and allowed to settle. A 0.1 mL aliquot of the appropriate suspensions (10^{-3} to 10^{-6}) were spread on SDA plates and incubated at 25 °C for 4 days. Counts were taken from the plates as colony forming units/g. The fungal isolates were characterized by slide culture and microscopic techniques and identified by the schemes of Tsuneo [16].

Germination Toxicity Test for Remediated soil

Toxicity of the remediated soil was assessed using germination test of Jaqueline *et al.*, [17]. *Cucumis sativus* (cucumber) was used in this study owing to its sensitivity to hydrocarbon in soil. Briefly, thoroughly mixed treated soil samples were placed in 100 × 15 mm Petri dishes. Ten viable seeds of *Cucumis sativus* were placed evenly throughout each Petri dish and covered with dry sand. Three replicates of the samples were prepared and 10 mL distilled water was sprinkled daily. Soil neither polluted nor amended served as the positive control while polluted soil without amendment served as the negative control. At the end of 21 days, the number of seedlings that emerged from the surface of the sand were counted and recorded. Their root lengths were measured to the nearest mm using a meter rule. Germination index of cucumber seed on the remediated soil was calculated using the formula of Millioli *et al*. [18]

$$GERMINATION\ INDEX(\%) = (\%\ seed\ germination \times \%\ root\ elongation) \div 100$$

$$\%\ Seed\ Germination = \frac{Number\ of\ seed\ germination\ on\ polluted\ soil}{Number\ of\ seed\ germination\ on\ positive\ control\ soil} \times \frac{100}{1}$$

$$\%\ Root\ Elongation = (GERm \div GERCm) \times 100$$

Where, GERm = root length of seedling that germinated on treated soil, GERCm = root length of seedling that germinated on control soil.

Statistical Analysis

The data obtained in the present study were subjected to analysis of variance (ANOVA). Relationship between variables and comparison of means of the different treatments were tested for level of significances at $P \leq 0.05$ using least square difference and post-hoc multiple comparison tests. The data analysis was performed using SPSS.

III. RESULTS

Physicochemical Properties of Soil and Potato Skin

The physicochemical properties of the soil and potato skin used in this study are presented in **Table 1.** The soil textural class was clayey loam and it had nitrogen (0.15%), organic carbon (2.49%), phosphorus (10.64%), moisture (15.38%), pH (7.03%), sand (31.5%), silt (19.75%) and clay (48.75%). The soil used for bioremediation had C: N ratio of 16:6. The potato skin had nitrogen content of 0.602%, phosphorus content 24.08%, organic carbon content 29.93%, moisture content of 48.68% and pH values of 6.8.

Table 1. Physicochemical properties of soil and organic wastes used for bioremediation.

	Nitrogen (%)	Phosphors (%)	Moisture content (%)	Organic carbon (%)	pH	Sand (%)	Silt (%)	Clay (%)
soil	0.15±0.02	10.64±1.50	15.38±0.30	2.49±1.10	7.03±1.15	31.5±0.6	19.75 ± 1.95	48.75 ± 2.75
Potato skin	0.602±0.1	24.08±2.0	48.60±3.5	29.93±0.91	6.8±0.49	-	-	-

Extraction Efficiency of Solvents for Crude Oil

The level of extraction of crude oil by three solvents namely: n-hexane, dichloromethane and diethylether eight hours post-pollution were 85.5%±0.07, 86.7% ±0.76 and 89.0% ±1.97

Determination of TPH Loss (Bioremediation)

The level of crude oil loss in both the control soil and polluted soil amended with 5% PS over a 42-day period is presented in **Figure 1**. Percentage oil loss in the amended soil ranged from 27.18% to 60.77%. Oil loss in the control option ranged from 15.67% to 17.56%.

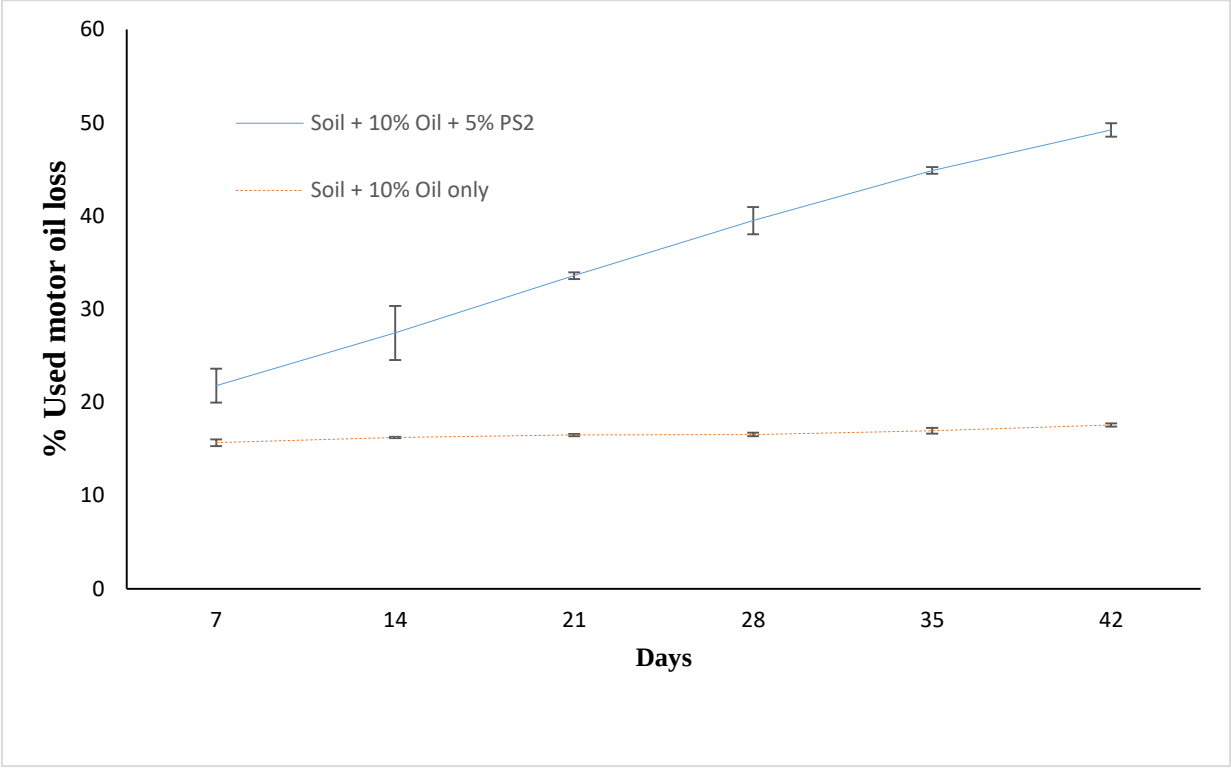

Fig.1: Bioremediation in soil polluted with 10% used motor oil and amended with 5% (w/w) PS. Error bars indicate standard errors (n = 3).

Figure 2 shows the level of oil loss in both the control soil and polluted soil amended with 10% PS. Oil loss in the PS-amended soil ranged from 31.54% to 63.84% within the 42-day period.

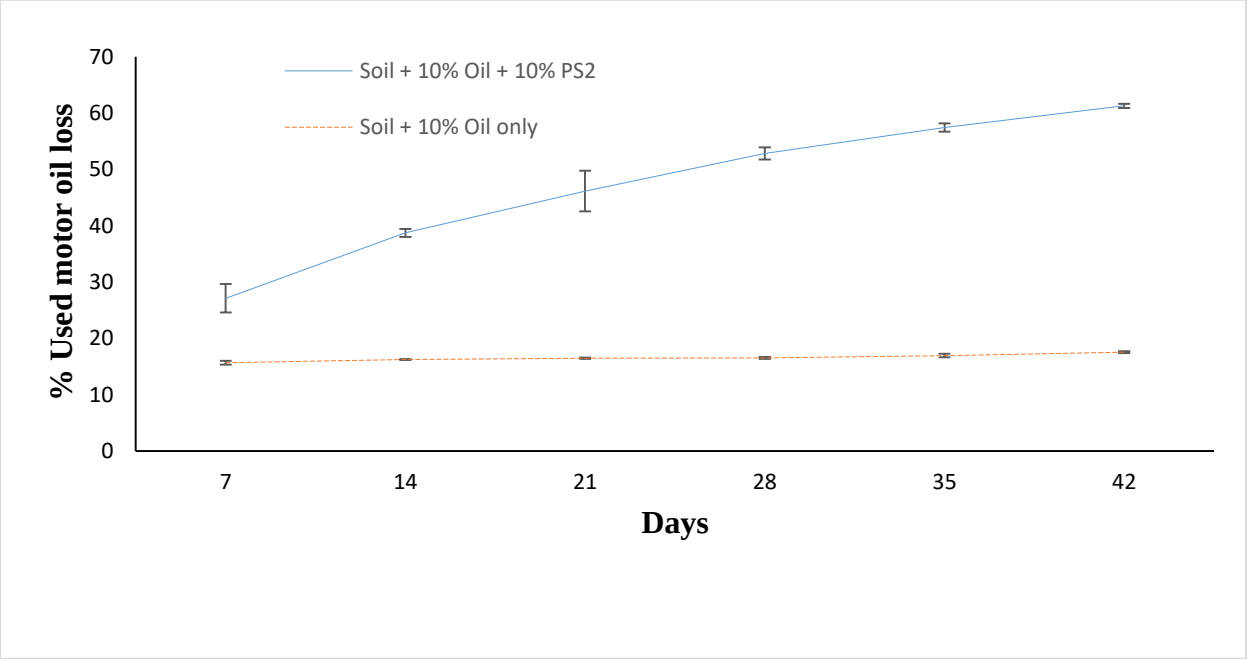

Fig.2: Bioremediation in soil polluted with 10% (v/w) used motor oil and amended with 10% (w/w) PS. Error bars indicate standard errors (n = 3).

The level of oil loss in the control and polluted soil amended with 15% PS over a 42-day period is presented in **Figure 3.** Percentage oil loss in the amended soil ranged from 40.52% to 73.85%.

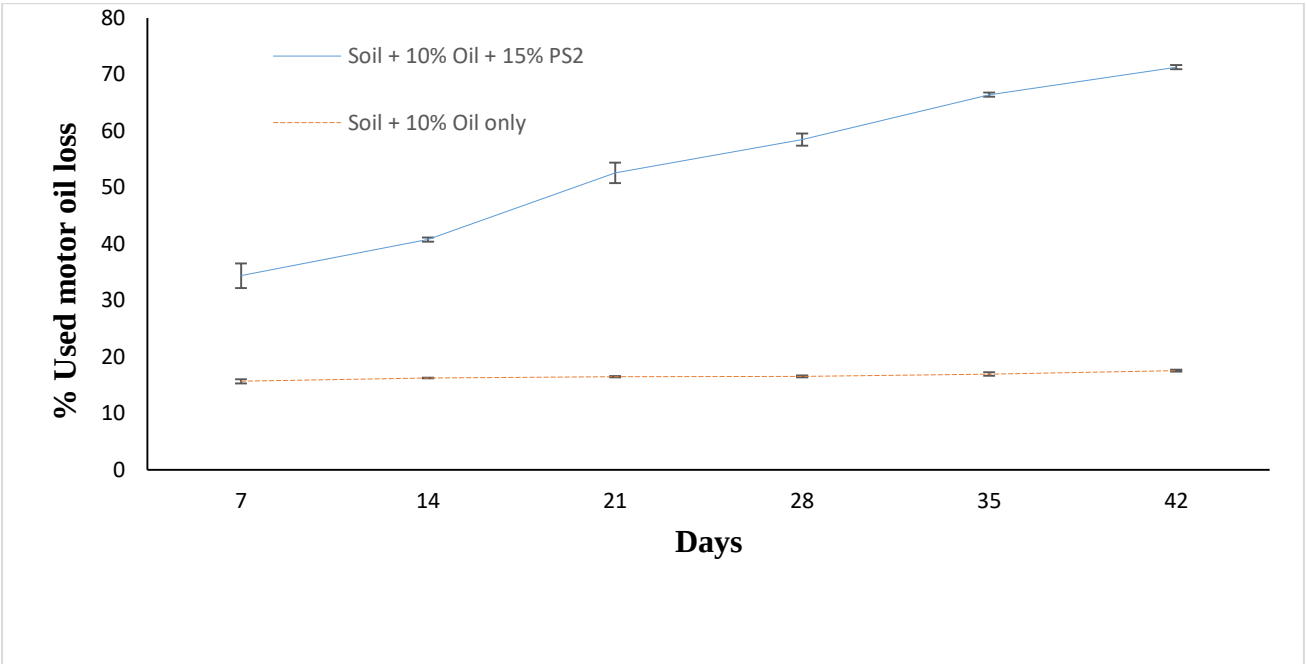

Fig.2: Bioremediation in soil polluted with 10% (v/w) used motor oil and amended with 10% (w/w) PS. Error bars indicate standard errors (n = 3).

Microbial Populations Recorded Throughout the Forty-Two-Day Period

Tables 2 shows the microbial populations in polluted control oil and polluted soil with three levels of amendment at day 0. Active aerobic heterotrophic bacterial colonies (AHB) were recorded in potato skin (PS)-amended soil, ranging from 9×10^7 to 17.2×10^7 CFU g^{-1} across all amendment levels. Unamended soil (control) gave AHB count 0.6×10^7CFU g^{-1} of soil. Similarly, hydrocarbon utilizing bacteria colonies (HUB) were recorded in PS-amended soil, ranging from 6.7×10^6 to 11.5×10^6CFU g^{-1} across all amendment levels. Unamended soil (control) gave HUB count of 0.2×10^6 CFU g^{-1} of soil. Furthermore, fungal population were recorded in PS-amended soil, ranging from 4.8×10^5 to 8.9×10^5 CFU g^{-1} across all amendment levels. Fungal count of $0.1 \times 10^5$was recorded in the unamended control soil.

Table 2: Microbial population on Day 0

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	9.0×10^7	6.7×10^6	4.8×10^5
Soil + 10% used motor oil + 10% potato skin	11.0×10^7	8.3×10^6	5.3×10^5
Soil + 10% used motor oil + 15% potato skin	17.2×10^7	11.5×10^6	8.9×10^5
Soil + 10% used motor oil only	0.6×10^7	0.2×10^6	0.1×10^5

Microbial counts recorded on day 7 for the control soil and polluted soil with three levels of amendment are presented in **Table 3**. Active aerobic heterotrophic bacterial colonies (AHB) were recorded in potato skin (PS)-amended polluted soil, ranging from 10.8-23.7 $\times 10^7$ CFU g^{-1} across all amendment levels. Unamended soil (control) gave AHB count 1.85×10^7 CFU g^{-1} of soil. Similarly, hydrocarbon utilizing bacteria colonies (HUB) from the PS-amended option ranged from 8.1-13.8 $\times 10^6$ CFU g^{-1} across all amendment levels. Unamended soil (control) gave HUB count of 1.4×10^6 CFU g^{-1} of soil. Furthermore, fungal population recorded in PS-amended soil ranged from 4.9×10^5 to 10.1×10^5 CFU g^{-1} across all amendment levels. Fungal count of 0.6×10^5 was recorded in the unamended control soil

Table 3: Microbial population on Day 7

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	10.8×10^7	8.1×10^6	4.9×10^5
Soil + 10% used motor oil + 10% potato skin	15.2×10^7	9.3×10^6	7.5×10^5
Soil + 10% used motor oil + 15% potato skin	23.7×10^7	13.8×10^6	10.1×10^5
Soil + 10% used motor oil only	1.85×10^7	1.4×10^6	0.6×10^5

Tables 4 shows the microbial populations in polluted control soil and polluted soil with three levels of amendment at days 14. Active aerobic heterotrophic bacterial colonies (AHB) were recorded in potato skin (PS)-amended polluted soil, ranging from 14.3×10^7 to 28.9×10^7 CFU g^{-1} across all amendment levels. Unamended soil (control) gave AHB count 3.7×10^7CFU g^{-1} of soil. Similarly, hydrocarbon utilizing bacteria colonies (HUB) were recorded in PS-amended soil, ranging from 9.8×10^6 to 15.3×10^6 CFU g^{-1}across all amendment levels. Unamended soil (control) gave HUB count of 2.9×10^6 CFU g^{-1} of soil. Furthermore, fungal population were recorded in PS-amended soil, ranging from 7.3×10^5 to 13.5×10^5 CFU g^{-1} across all amendment levels. Fungal count of 1.5×10^5 was recorded in the unamended control soil

Table 4: Microbial population on Day 14

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	14.3×10^7	9.8×10^6	7.3×10^5
Soil + 10% used motor oil + 10% potato skin	18.4×10^7	10.8×10^6	9.3×10^5
Soil + 10% used motor oil + 15% potato skin	28.9×10^7	15.3×10^6	13.5×10^5
Soil + 10% used motor oil only	3.7×10^7	2.9×10^6	1.5×10^5

Microbial counts recorded on day 21 for the control soil and polluted soil with three levels of amendment are presented in **Table 5**. Active aerobic heterotrophic bacterial colonies (AHB) were recorded in potato skin (PS)-amended polluted soil, ranging from 25.7-67.0 × 10^7 CFU g^{-1} across all amendment levels. Unamended soil (control) gave AHB count 5.9 ×10^7CFU g^{-1} of soil. Similarly, hydrocarbon utilizing bacteria colonies (HUB) from the PS-amended option ranged from 11.9-17.1 × 10^6CFU g^{-1} across all amendment levels. Unamended soil (control) gave HUB count of 3.7×10^6 CFU g^{-1} of soil. Furthermore, fungal population recorded in PS-amended soil ranged from 13.9 × 10^5 to 23.1 × 10^5 CFU g^{-1} across all amendment levels. Fungal count of 2.1 × 10^5 was recorded in the unamended control soil.

Table 5: Microbial population on day 21

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	25.7×10^7	11.9×10^6	13.9×10^5
Soil + 10% used motor oil + 10% potato skin	45.0×10^7	12.1×10^6	11.5×10^5
Soil + 10% used motor oil + 15% potato skin	67.0×10^7	17.1×10^6	23.1×10^5
Soil + 10% used motor oil only	5.9×10^7	3.7×10^6	2.1×10^5

Microbial populations recorded on day 21 for the control soil and polluted soil with three levels of amendment are presented in **Table 6**. Active aerobic heterotrophic bacterial colonies (AHB) were recorded in potato skin (PS)-amended polluted soil, ranging from 25.7-67.0 × 10^7 CFU g^{-1} across all amendment levels. Unamended soil (control) gave AHB count 5.9 ×10^7CFU g^{-1} of soil. Similarly, hydrocarbon utilizing bacteria colonies (HUB) from the PS-amended option ranged from 11.9-17.1 × 10^6CFU g^{-1} across all amendment levels. Unamended soil (control) gave HUB count of 3.7×10^6 CFU g^{-1} of soil. Furthermore, fungal population recorded in PS-amended soil ranged from 13.9 × 10^5 to 23.1 × 10^5 CFU g^{-1} across all amendment levels. Fungal count of 2.1 × 10^5 was recorded in the unamended control soil.

Table 6: Microbial population on day 28

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	28.3×10^7	13.1×10^6	22.6×10^5
Soil + 10% used motor oil + 10% potato skin	55.0×10^7	14.7×10^6	26.3×10^5
Soil + 10% used motor oil + 15% potato skin	89.0×10^7	19.1×10^6	27.4×10^5
Soil + 10% used motor oil only	7.2×10^7	4.1×10^6	4.4×10^5

Tables 7 shows the microbial populations recorded in all the amended microcosms and the unamended control option on day 35. Active aerobic heterotrophic bacterial (AHB) counts recorded in potato skin (PS)-amended polluted soil ranged from 57.0×10^7 to 98.0×10^7 CFU g^{-1} across all amendment levels. AHB counts in the control option were 9.9×10^7CFU g^{-1} of soil. Similarly, HUB counts recorded in PS-amended soil ranged from 16.7×10^6 to 21.1×10^6 CFU g^{-1} across all amendment levels. Unamended soil (control) gave HUB count of 6.3×10^6 CFU g^{-1} of soil. Furthermore, fungal population were recorded in PS-amended soil, ranging from 38.0×10^5 to 51.0×10^5 CFU g^{-1} across all amendment levels. Fungal count of 5.7×10^5 was recorded in the unamended control soil.

Table 7: Microbial population on Day 35

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	57.0×10^7	16.7×10^6	38.0×10^5
Soil + 10% used motor oil + 10% potato skin	63.0×10^7	17.1×10^6	45.0×10^5
Soil + 10% used motor oil + 15% potato skin	98.0×10^7	21.1×10^6	51.0×10^5
Soil + 10% used motor oil only	9.9×10^7	6.3×10^6	5.7×10^5

Microbial counts recorded on day 42 for the control soil and polluted soil with three levels of amendment are presented in **Table 8**. Active aerobic heterotrophic bacterial colonies (AHB) were recorded in potato skin (PS)-amended polluted soil, ranging from 53.0-106.0 $\times 10^7$ CFU g^{-1} across all amendment levels. Unamended soil (control) gave AHB counts of 10.7×10^7CFU g^{-1} of soil. Similarly, HUB counts from the PS-amended option ranged from 14.9-22.3 $\times 10^6$ CFU g^{-1}across all amendment levels. Unamended soil (control) gave HUB count of 7.1×10^6 CFU g^{-1} of soil. Furthermore, fungal population recorded in PS-amended soil ranged from 39.0×10^5 to 59.0×10^5 CFU g^{-1} across all amendment levels. Fungal count of 6.4×10^5 was recorded in the unamended control soil.

Table 8: Microbial population on Day 42

Soil preparations	Colony forming units/gram		
	AHB	HUB	Fungi
Soil + 10% used motor oil + 5% potato skin	53.0×10^7	14.9×10^6	39.0×10^5
Soil + 10% used motor oil + 10% potato skin	71.0×10^7	19.9×10^6	51.0×10^5
Soil + 10% used motor oil + 15% potato skin	106.0×10^7	22.3×10^6	59.0×10^5
Soil + 10% used motor oil only	10.7×10^7	7.1×10^6	6.4×10^5

Isolates Identified

The identity of hydrocarbon-utilizing bacteria isolated from both the control soil and amended soil throughout the 42-day period are presented in **Tables 9 and 10**. Six hydrocarbon-utilizing bacteria belonging to the genera *Bacillus*, *Arthrobacter*, *Rhodococcus*, *Corynebacterium*, *Pseudomonas* and *Acinetobacter* were predominantly isolated based on their Gram reaction and biochemical characteristics. Similarly, **Figure 4** presents the identity of the fungal isolates. The fungi include: *Aspergillus niger*, *Aspergillus* sp., *Pennicillium* sp., *Phialophora* sp., *Cladosporium* sp. and *Verticillum* sp.

Table 9. Morphological and microscopic characteristics of bacterial isolates.

Isolates	Morphological characteristics	Microscopic characteristics
SWO_a	Large, Round, Irregular, Flat, Milky, Smooth,	Small, Gram Positive, Rods
SWO_b	Small, Round, Irregular, Flat, Greenish, Smooth, Translucent	Small, Gram Positive, Staph
SPS_a	Large, Round, Irregular, Flat, Milky, Smooth,	Small, Gram Positive, Rods
SPS_b	Pin Point, Round, Entire, Flat, Greenish, Smooth, Opaque	Small, Gram Positive, Cocci
SPS_c	Small, Round, Irregular, Flat, Greenish, Smooth, Translucent	Small, Gram Positive, Staph
SPS_d	Large, Opaque, Irregular Creamy	Small, Gram Positive, Rods

SWO: soil with used motor oil, **SPS**: polluted soil amended with potato skin

Table 10. Biochemical characteristics of bacteria Isolates

Isolates	SWOa	SWOb	SPSa	SPSb	SPSc	SPSd
Gram reaction	+	+	+	+	-	+
Starch hydrolysis	+	-	+	-	-	+
Citrate utilization	-	+	-	+	+	+
Urease production	-	-	-	+	-	+
indole	-	-	-	-	-	-
H_2S	-	-	-	+	-	-

Methyl-red	+	-	+	+	-	-
Voges-Proskauer	-	-	-	-	-	-
Oxidase	-	-	-	+	-	-
catalase	+	+	+	+	+	+
motility	+	-	+	-	-	-
Spore formation	+	-	+	-	-	-
Probable identity	*Bacillus* sp	*Staphylococcus* sp	*Bacillus* sp	*Micrococcus* sp	*Staphylococcus* sp	*Arthrobacter* sp

Key: Negative (-); Positive (+); **SWO**: soil with used motor oil, **SPS**: soil amended with potato ski

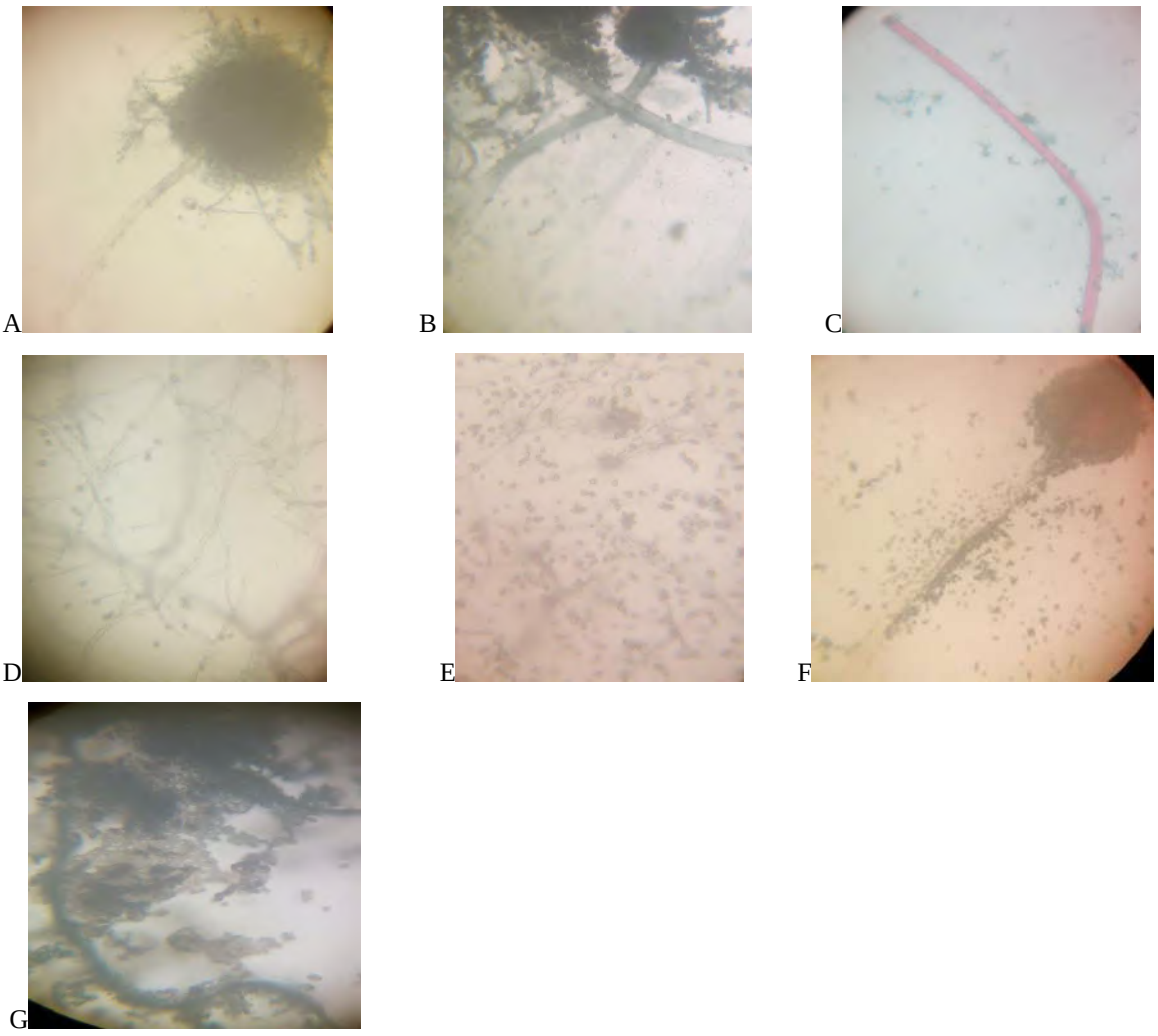

Fig.4: Fungal reproductive structures in Sabouraud dextrose agar. A: Filamentous fungus – Aspergillus niger; B: Conidiophores with spore masses of Cladosporium sp.; C: Pink conidiophore and spores of Fusarium sp.; D: Conidiophore, phialides and conidia of Phialophora sp.; E Penicillium sp.; F: Aspergillus fumigatus; G: Thick walled resting cells of Verticillum sp.

Seed Germination Toxicity and Germination Index

The results of germination toxicity test with *Cucumis sativum* for positive control soil, negative control soil and polluted soil across all levels of amendment are presented in **Table 11**. Percentage SG ranged from 50 to 75 across all amendment levels, %GR ranged from 65.65-92.16 across all amendment levels while GI values ranged from 38.83 to 69.46 across all amendment levels. Positive and negative controls had GI of 100 and 3.80 respectively.

Table 11: Seed Germination Parameters of Control and Amended Soils

Soil preparations	SG	GR(cm)	%SG	%GR	GI
Soil + 10% used motor oil + 5% potato skin	4.00	1.51	50.00	65.65	38.83
Soil + 10% used motor oil + 10% potato skin	5.00	1.67	62.50	72.61	45.38
Soil + 10% used motor oil + 15% potato skin	6.00	2.13	75.00	92.16	69.46
Soil + 10% used motor oil (negative control)	1.00	0.70	12.5	30.43	3.80
Soil with no oil contamination (positive control)	8.00	2.30	100.00	100.00	100.00

SG: Number of seeds that germinated; GR: Root length; GI: Germination index

IV. DISCUSSION

A review of studies on bioremediation of hydrocarbon contaminated sites reveals a growing concern by scientists to understand better ways of treating soils contaminated with petroleum products. In the present study, the remediation of used motor oil-contaminated soil by the use potato skin amendment to stimulate microbial activity was explored. A good number of microorganisms, including bacteria and fungi, could be found in the soil. In UMO-polluted soil, bacteria and fungi are continuously exposed to UMO, giving rise to survival of bacteria and fungi that can utilize the same. The data presented in this research were limited to laboratory experiments. The results from our study showed significant reduction in the level of used motor oil contamination in the potato skin-amended soil

In the present study, potato skin had higher nitrogen content than the experimental soil (**Table 1**). Nitrogen and phosphorus have been reported as essential nutrients for bioremediation of petroleum hydrocarbons in the soil [19]. In other words, as bioremediation progresses in the absence of external nutrient sources such as nitrogen, carbon, phosphorus etc., the microorganisms will utilize available nutrients in the soil to a point of depletion and the nutrients becomes limiting. The soil pH (7.03±1.15) **(Table 1)** was within the acceptable limits required for effective biodegradation [20].

The result of the extraction efficiency experiment clearly revealed that diethylether was the best choice in the extraction of used motor oil among the other two solvents, namely dichloromethane and n-hexane. This is due to the highest percentage extraction (89%) observed with diethylether.

In the present study, reduction in Total Petroleum Hydrocarbons (TPH) increased appreciably as the weeks of biodegradation increased. Oil loss increased notably (between 27.18% and 73.85%) throughout the 42-day period across all amendment levels **(Figure 1-3)**. Highest oil loss was observed in the highest amendment level (15%) **(Figure 3)**. The observed enhancement in oil loss at 15% amendment level was probably due to the enhanced level of nutrients at that level of amendment. It was reported that TPH removal always increases as the days of incubation increases [21]. In a similar vein, Abdulsalam [22] also reported an increase in removal of TPH in soil contaminated with used motor oil as the incubation period was elongated.

Oil loss in the control option was also progressive throughout the study period but was much slower **(Figure 1-3).** Mechanisms such as photodegradation, volatilization, sorption and bioattenuation by the indigenous hydrocarbonoclastic flora might have played some contributory roles to the observed trend in the control microcosm. Similar works recorded low TPH loss in the control option [23, 24]

Critical evaluation and assessment of reports on biostimulation-based bioremediation processes reveal some level of inconsistency. While some have demonstrated direct relationship between TPH loss and organic amendment [25, 26], a scenario where natural attenuation in the unamended soil was more successful than biostimulation has been

reported [27]. Also, there was no significant effect of nutrient amendment in the work carried out by Seklemova [28]. It stands to reason therefore, that soils have varying degrees of microbial potentials as touching degradation of hydrocarbon pollutants.

Microbial growth and activities can be used as a probe for impact of organic wastes [23]. In the present work, the microbial counts study revealed that AHB, HUB and fungal populations increased appreciably in each successive week, AHB populations being the greatest in each week. It was also observed that HUB populations were greater than their fungal counterparts all throughout the study period (**Table 6**). Among the bacterial groups, HUB was found to be lower than the AHB. It might be deduced from the observed trend that HUB are a group of AHB that evolved due to incessant hydrocarbon spills. In a similar manner, it was noted that fungal groups were lower in number than their hydrocarbon-degrading bacterial counterparts (**Tables 2-8**). Chikere *et al* [29] argued that even while it has been generally accepted that fungi and bacteria are the major microbial groups involved in hydrocarbon remediation, bacteria have been found to be more versatile and therefore participate more frequently in several microbial remediation processes. Higher TPH loss and microbial levels were observed in the amended options. Similar trend has been widely documented [30, 31, 32]. In another sense, the observation of higher microbial counts and TPH loss in the amended options might be due to the diverse groups of microbes associated with potato skin with innate hydrocarbon-degrading abilities.

Tentative identification of the hydrocarbon-utilizing bacteria isolated in the present study revealed the presence of bacteria belonging to the genera: *Bacillus, Arthrobacter, Rhodococcus, Corynebacterium, Pseudomonas, Staphylococcus* and *Acinetobacter*. Earlier research documented these bacteria as having potential hydrocarbon-remediation attributes [33]. Among the bacteria, *Bacillus* was predominantly isolated in this work **(Tables 9-10)**. The ability of *Bacillus* isolated from Nigerian soil has consistently been observed and attributed to competent hydrocarbon- degrading enzyme system of the organism, its ability to form spores and emulsify crude oil [26]. Similarly, the fungi isolated were tentatively identified as *Aspergillus niger*, *Aspergillus* sp., *Pennicillum* sp., *Phialophora* sp., *Cladosporium* sp. and *Verticillum* sp. **(Figure 4)**. Adekunle and Adeniyi [34] reported species of *Penicillum* and *Aspergillus* that degrade kerosene, spent engine oil, unspent engine oil, diesel and extracted oil from *Treculia africana* seed.

Cucumber seed is sensitive to toxic chemicals (mostly petroleum hydrocarbons) and it is an important agricultural crop, which led to its wide use for toxicity test and as a bioindicator [17]. Millioli *et al.* [35] reported a decrease in the number of germinated seeds with 10% petroleum contamination in the soil. Germination index (GI) of soil treated with 15% PS gave the highest value of 69.46 (**Table 11)**. However, the GI of plants grown on untreated contaminated soil was very low, signifying low bioremediation in the treatment option. Hydrocarbons may affect root surface, preventing or reducing gas and water exchange and nutrient absorption. Hydrocarbons may also enter the seeds and alter the metabolic reactions and kill the embryo. Hydrocarbons damage cell membranes and reduce the metabolic transport and respiration rate [36, 37]. In the present study, variations in plant growth parametres in the different soil preparations have shown that soil quality can affect productivity. In a 126-day study using soy cake, potato skin, and tea leaf amendments, Agamuthu and Dadrasnia [25] reported higher percentage seed germination of 90%, 70%, and 60%, respectively with seeds of lettuce (*Lactuca sativa L.*) in just seven days after treatment. Ogboghodo [36] and Oleszczuk [37] reported that low percentage germination and low germination index is sequel to low biodegradation of oil or short treatment of oil-contaminated soil. In the present study, it took the cucumber seeds a minimum of 21 days to germinate instead of the normal range of 7-10 days; this signifies that cucumber seeds are very sensitive to used motor oil. Growth of all seeds planted was recorded in the positive control while a lower percentage germination recorded in the negative control signified that germination of seeds can go undisturbed in a hydrocarbon-free soil. There was a significant difference in bioremediation level between the control and amended soil even at 5% amendment level.

V. CONCLUSION

Used motor oil pollution of soil proved to negatively alter the soil quality, depressing aerobic heterotrophic bacterial counts and encouraging the proliferation of oil utilizing bacteria in the soil. Amendment of UMO-polluted soil with potato skin caused proliferation of oil-degrading microbes and enhanced microbial degradation of used motor oil in the soil. Potato skin might have provided alternative source of N and P, to stimulate microbial activity. The study therefore shows the viability of using potato skin amendment in remediating hydrocarbon-contaminated soil. Potato skin

therefore affords an alternative method in removing used motor oil contaminants from soil.

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Viability of Municipal Solid Waste as a source for Bioenergy products production

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Abstract*— *Energy is an important requirement for population growth, technological progress and urbanisation. Worldwide energy demand has been projected to increase 5-fold by 2100. Fulfilment of these energy requirements cannot be solely reliant on fossil fuels, such as oil, coal and natural gas, on account of their adverse environmental impacts and concomitant depletion of natural resources. As a result multiple approaches for generating alternative energy are being explored globally.In this review paper, focused on the viability of waste especially MSW being a source for bioenergy products such as methane gas, bio-enzyme, biofuel and bio-fertiliser production. This review also focuses on the environmental impacts of MSW, the effect of MSW pre-treatments and properties (physical and chemical) on bioenergy products production.

Keywords*— *MSW; pre-treatment; MSW management; Solid State Fermentation (SSF); recycling;Biogas; Methane gas; Anaerobic digestion (AD); biofuel;ethanol; butanol and bio-fertiliser.

I. INTRODUCTION

Environmental impact of Municipal Solid Waste (MSW)

The earth's climate is changing, with temperatures rising since the beginning of the twentieth century partly due to an increase in atmospheric concentrations of greenhouse gases. Climate change has become a long term concern and as a result production of bio energy is considered to be one solution for solving environmental issues such as water, soil and air pollution, as well as decreasing reliance on fossil fuels.

There are many resources which are available for producing bio energy, such as agricultural crops and their waste, animal waste, food processing and MSW. These are considered as potential renewable and sustainable energy sources. Generally, MSWs are considered to be one of the most sustainable resources world-wide[1]. Additionally, MSW presents an environmental problem in relation to its disposal. Furthermore, MSW treatments play a critical role in producing bio-energy in the form of high quality gases; biofuels and fertilisers. Presently there are many available technologies applying for bio-energy production, such as Anaerobic Digestion (AD), incineration, Refuses Deprival Fuel (RDF) and fermentation for biofuel productions [2].

MSW is a complex waste material whose composition is heterogeneous in nature, within MSW some of the components are stable, while others degrade as a result of biological and chemical processes causing potential pollution problems [3]. Waste generation is becoming increasingly significantly (Fig 1.1) which is leading to increasing pollution problems. For this reason, Solid Wastes Management (SWM) is an important consideration in order to decrease the effect of pollution.

In most countries, solid waste landfill is the most common means of disposal. Landfill sites are unsightly, unsanitary, generally smelly, and attract animals and insects [4]. To overcome the problem of MSW, incineration is wildly applied; incineration produces a safe substrate called ash, which can be used for further applications[5, 6]. Unmanaged MSWs have various impacts on the environment and on human health. For instance, water may become polluted by leachate if the leachate enters surface and ground water before sufficient dilution. Presence of leachate in the water may lead to serious pollution which affects animal and human life[7]. Personal use of water polluted by MSW for bathing, food irrigation and drinking water can expose individuals to disease causing organisms and other contaminants [8].

In regards, atmospheric pollution, MSW have been shown to emit Green House Gases (GHG) during the decomposition of solid waste when present in the landfill [9]. Managing MSW can lead toa decrease in GHG emissions as shown in a study carried out in Europe (EU)

where overtime total annual net CO_2production decreased(Fig 1.2). In addition, the World Health Organization (WHO) estimates that around a quarter of diseases affecting human health occur due to prolonged exposure to environmental pollution [10].

Generally, bio-energy production has a negative impact on living organisms due to the increase in erosion, depletion of soil nutrients and soil quality. These problems are related to the cultivation of annual crops; a further impact is in the use of pesticides and fertilisers. These agriculture inputs can affect people's health by leaching residues into ground water [11].

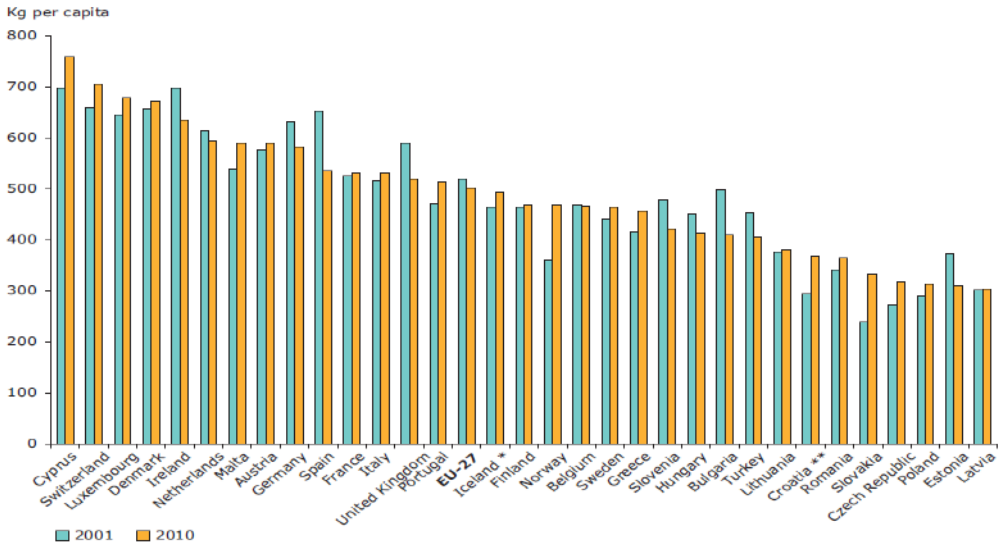

Fig.1.1 Municipal wastes generated per capita, 2001 and 2010[12, 13]

Note: (*) 2008 data used for 2010. (**) 2004 data used for 2001. According to Eurostat the comparability of the data over time is high. However, some breaks in the time series are documented, which can influence the comparability between countries and within a country. Generally, the quality of the data has improved during the period 2001–2010.

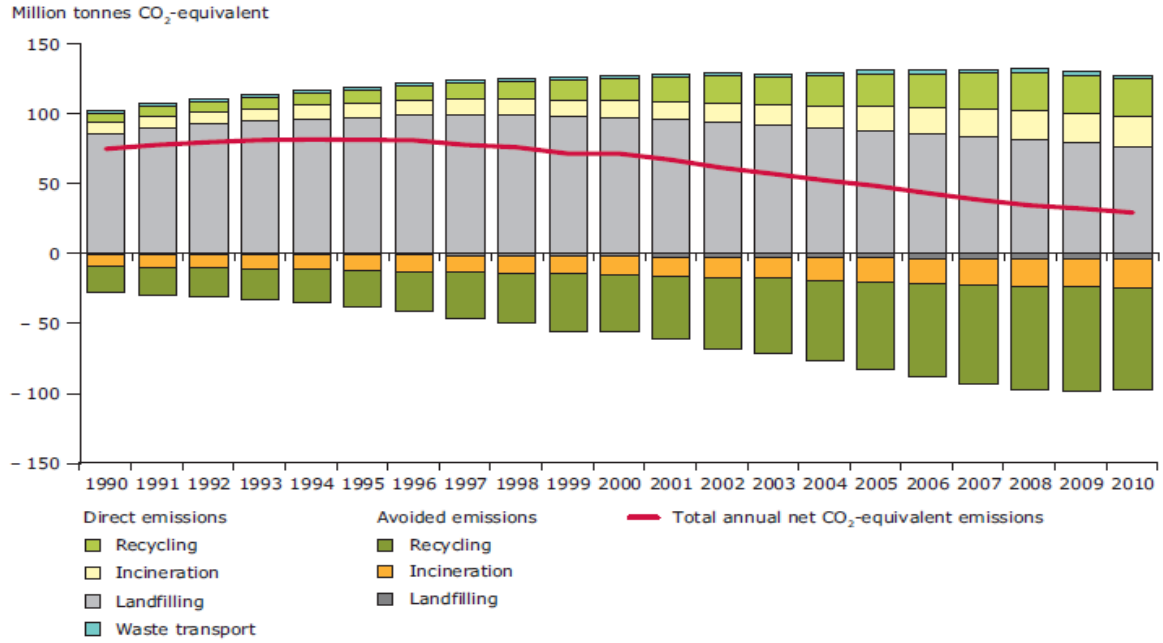

Fig.1.2 GHG emissions from municipal waste management in the EU, Switzerland and Norway[14]

Note: Excluding Cyprus due to lack of data. GHG emissions before 1995 are calculated based on backcasted waste data.

The concentration of CO_2 in the atmosphereis responsible for global worming hadby 2001 risen to 391ppm; an increase of about 6% compared to records forthe year 2000. Comparing GHG production, in particular CO_2, with the world total population, the 2010 world energy statistics [15] show that 44% of total CO_2 emission comes from 17% of the world total population (developed nations) while the remaining 83% of the world population (developing and least developed) contribute half of the total emissions [16](see Fig 1.3).

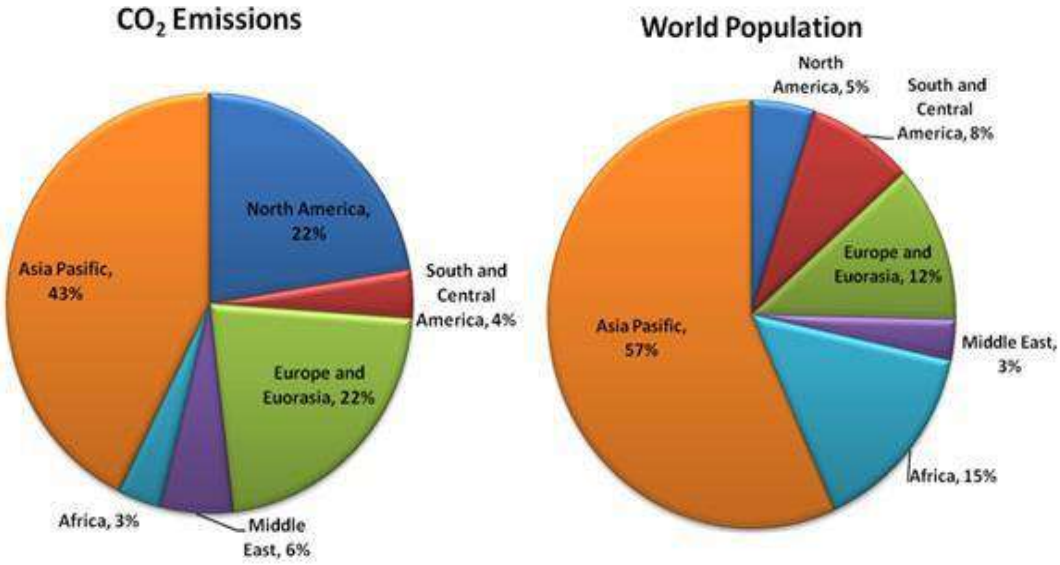

Fig.1.3 Comparison of World Population and CO_2 emission[17].

II. CURRENT AND FUTURE APPLICATIONS FOR MSW

Nowadays industrial chemical synthesis need to consider impacts including energy use, economic and effects on the environment. So any commercial products must be produced with minimum energy requirement. One way to satisfy the above constraints is by using a biochemical processes. These processes can be environmentally friendly, cost effective and carried out at ambient conditions [18]. Examples are methane or biofuel production [19] and bio-compost production from MSW [20].

2.1 Methane gas production by anaerobic digestion

AD is a process that has attracted increasing attention in both developing and developed countries as a promising approach for the conversion of organic waste into biogas. AD is a biological treatment method that relies on microbial activity to digest the waste [21]. It was originally used to manage the accumulation of organic wastes and/or for organic fertiliser production, but the emphasis is now shifting to renewable energy generation. These facilities generally treat organic materials which are abundant in their geographical locations. Consequently, waste from farm animals is the predominant feedstock for AD in China, India and North America, wastewater in North America, while MSW and industrial food processing wastes are utilised in Europe, [22, 23]. There were around 120 plants operating in Europe between 1998 and 2008, with a total operational capacity of around 4.6 million tonnes per annum, with the highest production in Germany Fig 2.1. The EU was the highest biogas producer in the world in 2012 and is predicted to maintain this position until 2022 (Fig 2.2.)

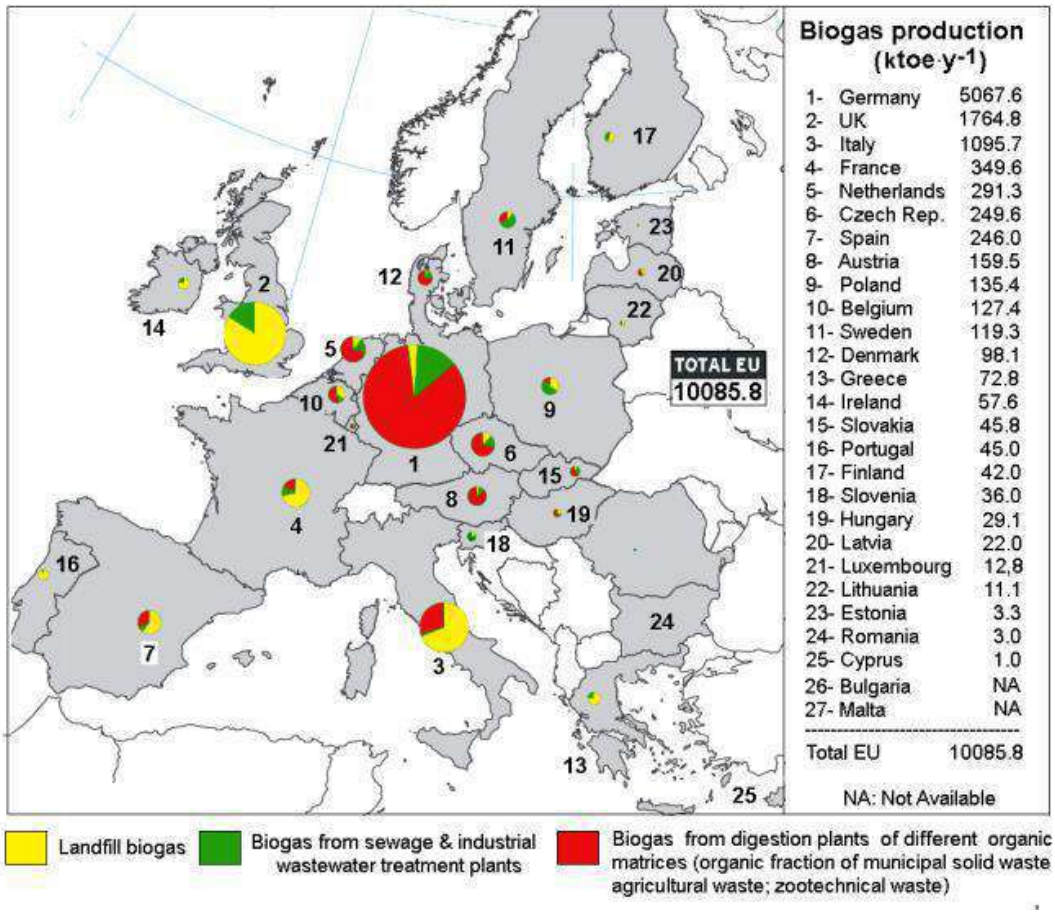

*Fig.2.1 Primary energy production of biogas in the European Union in 2011 (ktoe /y)***[24].**

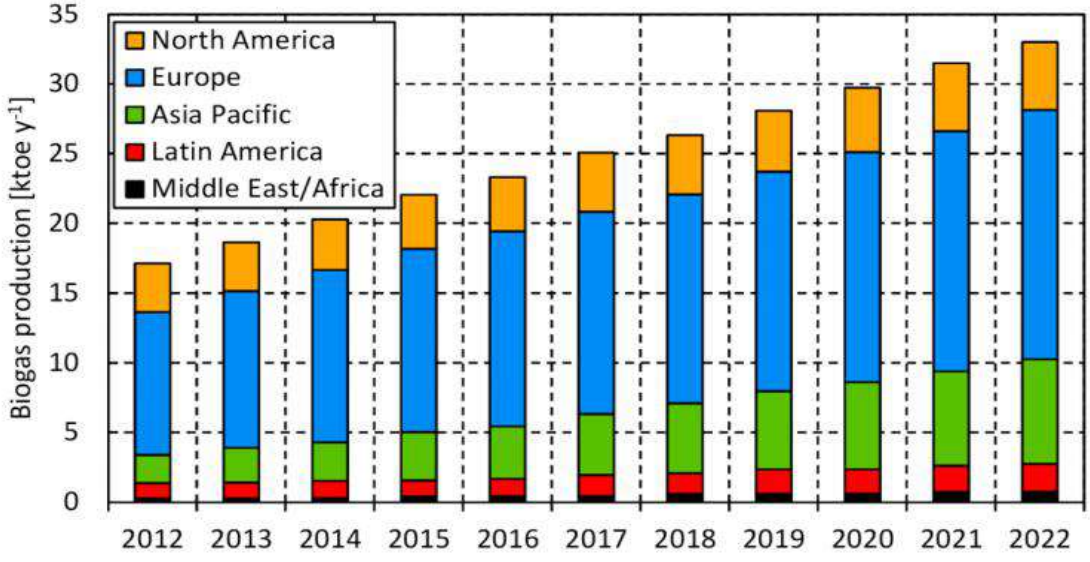

*Fig.2.2 Biogas production at 2012 and predicted trend to 2022 in different areas of the world***[25].**

Second generation biofuels

Bio-energy is energy which is derived from biomass, bioenergy production is an attractive, controllable and storable form of renewable energy [26]. Bio-energy can be generated by biological, chemical or physical processes. Energy ultimately derived from sunlight is stored as chemical bonds between carbon, hydrogen and oxygen atoms; these bonds can be broken down by digestion, combustion or decomposition releasing the stored energy [27].

Substrates require pre-treatment for biofuel production. The best and most effective pre-treatments are those that require no reduction in particle size, preserve the pentose (hemicellulose) fraction, avoid formation of possible inhibitors of hydrolytic enzymes and fermenting microorganisms, minimise energy use, have low running costs (operating costs, capital costs, and biomass costs), low catalyst cost, consume of little or no chemicals and which use cheap chemicals[28]. Pre-treating lignocellulosic material aims to produce a more reactive material than the original material; this process can produce soluble fermentable sugars [29].

Several methods have been introduced for pre-treatment of lignocellulosic material prior to enzymatic hydrolysis or digestion. These methods are classified into: physical pre-treatment, physio-chemical pre-treatments, chemical pre-treatments, and biological pre-treatments [30, 31].

Physical pre-treatments

Physical pre-treatments, includes size reduction such as chipping, shredding, grinding, and milling. These methods have been used to enhance the digestibility of lignocellulosic biomass [32]. Harvesting and preconditioning reduces lignocellulosic biomass from logs to coarse particle sizes of between10–50mm.Chipping reduces the biomass particle size to 10–30mm, however, the process reduces heat and mass transfer limitations. Grinding and milling can further reduce particle size to 0.2–2mm and these processes can start to have an impact on the crystallinity of cellulose.

Research has revealed that reducing biomass particle size below 40 mesh (0.4mm) has little effect on rates or biomass hydrolysis yields[33]. Reduction of particle size hasn't been studied widely in terms of hydrolysis [34], but some studies have shown that milling increases biogas, bioethanol and bio-hydrogen yields. Using milling will increase the cost of production, however, milling is viewed as being economically feasible [29]. **Thermal pre-treatments**are applied in order to solubilise hemicellulose, thereby improving rates of hydrolysis of lignocellulose material [35]. There are various thermal pre-treatment methods such as use of steam (~ 240°C and high pressure for a few minutes) [29], steam explosion (a rapid release in pressure which causes a disruption in the structure of the material) [29], liquid hot water treatment (where water is maintained as a liquid at high temperatures (160 to 230°C) and under high pressures (>5 MPa) [36-38].

As the temperature used in the hydrolysis increases above 150–180ºC, hemicellulose and lignin become solubilised [39]. There are two main components of hemicelluloses (xylan and gluco-mannans), the xylans are the least thermally stable when compared with gluco-mannans. If temperatures during pre-treatment exceed 180ºC, an exothermal reaction (probably solubilisation) of the hemicellulose begins [40], the thermal reactivity mainly depends on the composition of the lignocellulosic biomass and has an influence on the temperature at which this exothermal reaction begins [41].

Generally, during thermal processing, the hemicellulose portion of the plant cell wall becomes hydrolysed forming acids, presence of these acids catalyses the further hydrolysis of the hemicellulose [42]. Furthermore, thermal processes can cause an increase in the Crystallinity Index (CrI) of cellulose, though no increase was observed when the CrI was already high [43].

The thermal pre-treatments also produce phenolic compounds as a result of the solubilisation of lignin, these phenolic compounds have been shown to be toxic or inhibitory to the growth of bacterial, yeast and methanogens/archae[44]. Furthermore, if these phenolic compounds are not removed quickly they have been shown to re-condense as a precipitate onto the biomass [45]. Use of severe pre-treatment conditions enhances the condensation and precipitation of solubilised lignin compounds [46].

Steam pre-treatment is characterised by the use of a large vessel, steam at temperatures up to 240ºC and high pressure, moisture content of the biomass during pre-treatment with steam is an important factor, for example the higher the moisture content, the longer the optimum time required for steam pre-treatment [47]. Steam pre-treatment has been shown to solubilise a fraction of the hemicelluloses, this process is referred to as 'auto-cleave'. A common term used in steam pre-treatments is the so called 'severity factor' (log R0), which is a measure for the severity of the pre-treatment [29]. Steam-explosion is the most commonly used pre-treatment method, the process includes injecting high pressure saturated steam into a reactor, leading to the temperature rising to 160-260ºC. Pressure is suddenly reduced and the biomass undergoes an explosive decompression leading to the destruction of the

fibre structure, decreasing crystallinity of the cellulose, and increasing the surface area substrate [48].

Liquid Hot Water (LHW) biomass pre-treatment is a hydrothermal process, which does not require addition of chemicals, the process uses water under high pressure. This process has been shown to penetrate into the biomass, hydrate cellulose, and remove hemicellulose and part of the lignin, this makes the process cheap and more industrially relevant [49]. Use of hot water also reduces the requirement for reducing the size of the lignocellulosic prior to pre-treatment and produces lower amounts of neutralization residues. In this process, hemicellulosic carbohydrates are dissolved as liquid-soluble oligosaccharides and can be separated from insoluble cellulosic and lignin fractions [28]. This process increases enzyme accessibility by increasing surface area of the cellulose [50]. Pre-treatments with steam and LHW are both hydrothermal pre-treatments characterised by higher pentose recovery and lower formation of inhibitory components [51].

Mechanical pre-treatment (MPT)MPT is a process which includes waste sorting and homogenisation, and is followed by biological treatment [52]. This method is reliant depends on stressing the substrate cell wall without addition of any chemical substances [53, 54]. The MPT can break down the crystalline structure of cellulose; thus increasing the reactant surface area following fine milling (nano-milling) [48]. Other pre-treatments which use mechanical treatment are: High Pressure Homogenisation (HPH), stirred ball mills, and the jetting and colliding method [55, 56].

Autoclaving is a heat treatment, the autoclave is an instrument which uses relatively high temperatures, and pressure. This process has been applied previously to sterilise hospital wastes and some animal wastes [57].The process as applied on MSW is a relatively recent innovation and the commercial process is shown in Fig 2.3.

The main reasons for autoclaving unsorted MSW includes destroying bacteria, reducing the size of waste by 60%, reducing moisture content, removing recyclable materials from the waste stream and, finally, increasing the quality of recyclable metals (by stripping away label glue from food cans and heat shrink packaging). However, heat can have an adverse effect on some recyclable plastics, such as polyethylene terephthalate (PET) and high density polyethylene (HDPE) [58, 59].

The process consists of collecting waste from resources, followed by injecting unsorted MSW automatically into the autoclave [60]. To run any autoclaving method, some points need to be taken into consideration such as pressure (6.2 bar is maintained for between thirty and sixty minutes). Aeration can be supplied via a blower directed from the bottom through the material, and gasses are collected at the top in order to analyse them. Steam is injected at pressure and the temperature increased to 130^{o}C. This temperature is considered to be the optimal temperature for pre-treatment of total solids [61].

Chemical pre-treatments

There are many chemical substances which can be used for the pre-treatment of biomass such as oxidizing agents, alkali, acids and salt [62].Acid hydrolysis or pre-treatments, is one of the most common methods used for the pre-treatment of lignocellulosic biomass to attain higher sugar yields [63]. The main goal of this method is to solubilise the hemicellulosic fraction of the biomass, which increases the accessibility of the enzymes to the cellulosic fraction [64].

Either dilute or strong acid can be used for this type of pre-treatment to hydrolyse hemicellulose and solubilise or precipitate lignin [29]. Studies have shown that 0.5% H_2SO_4 is an optimal acid concentration for treatment of waste from vegetables and rice straw [65]. While, higher acid concentrations of up to 2.5 M are capable of separating lignin and other organic components [66, 67].

Weak acid hydrolysis (dilute acid treatment), the most efficient pre-treatment for lignocellulosic substrates with low lignin content is the use of dilute acid, which offers a good sugar recovery. The aim of this process is to remove hemicelluloses thus increasing porosity and as a result improving enzymatic digestibility [68]. Some disadvantages are the further degradation of hemicelluloses sugars which can be corrosive and degraded further to furfural and hydroxymethylfurfural (HMF), presence of these compounds can be inhibitory in microbial fermentations [35]. Indeed for some years dilute sulphuric acid has been added to biomass to manufacture furfural commercially [69]. The production process hydrolyses hemicelluloses to xylose which is then condensed into furfural, which is recovered by distillation [70].

Various dilute acids can be applied for pre-treating different lignocellulosic substances including sulphuric acid [71], nitric acid [72], hydrochloric acid [73], phosphoric acid [74], peracetic acid [75] and oxalic acid [76].

Among these, the most commonly applied is dilute sulphuric acid due to its availability, cost, safety and low environmental concerns [77]. Pre-treatment with sulphuric acid helps to achieve high yields of xylose from xylan[36].and increases the enzymatic digestibility of cellulose [78]. However, use of sulphuric acid produces

inhibitors such as furfural [79], dilute acid pre-treatments have also been found to be suitable for a wide range of feed stocks including softwood, hardwood, herbaceous crops, agricultural residues, waste paper and MSW [80].

However, use of dilute acid has some drawbacks such as corrosion, the need for neutralisation before the fermentation process, formation of degradation products and the acids or chemical price should be taken into consideration [81]. Dilute acid pre-treatments can increase the cellulose conversion by enzymes to sugar but doesn't fully remove lignin which precipitates on the cellulose surface and may inhibit the hydrolysis process [82]. To decrease the negative impact of lignin on the Enzyme Hydrolysis (EH) process, some studies have added surfactants such as Tween-80, dodecylbenzene sulfonic acid and polyethylene glycol 4000 (PEG, 4000), to acid pre-treated corn Stover biomass at 140–220°C.The presence of these surfactants enhances lignin removal and improves the digestibility of the cellulose by increasing the hydrophobicity of the biomass [83].

To further improve digestibility, dilute acid pre-treatments can be combined with other pre-treatments such as combining acid and alkaline pre-treatments (strong acid–strong alkali or weak acid–weak alkali), these combined pre-treatments have been shown to remove most of the non-cellulosic materials [84]. Generally there are two types of weak acid hydrolysis:

- High temperature and low-solids loading (T>160°C, 5-10% wt substrate concentration).
- Low temperature and high-solids loading (T≤160°C, 10-40% substrate concentration) [35].

Strong acid hydrolysis; Concentrated H_2SO_4 and HCl have been used for treating lignocellulosic substrate due to their ability to directly hydrolyse cellulose and thus not require any use of enzymes [85]. This method has some advantages such as high monomeric sugar yield and mild temperatures are required. However, drawbacks for this process are the corrosive nature of the acid and the need to recycle acid in order to lower costs, toxicity, and the requirement for expensive construction materials [86]. Some companies have commercialised the use of strong acids for microbial fermentation purposes [35].

Alkaline hydrolysis or pre-treatment, for alkaline pre-treatments, the most common chemicals used are calcium and sodium hydroxide for solubilising lignin [36]. Sodium hydroxide (NaOH) is mainly used because it is a safer chemical substance and can be recycled [87]. However, it is expensive and needs to be removed because of salt production [88]. This method has not been applied industrially [89]. Sodium hydroxide has received the greatest attention due to its outstanding delignification capacity which is essential to achieve high biomass digestibility [90]. The main goal of alkaline pre-treatment is to increase the internal surface area of the lignocellulosic material due to swelling induced by the alkali [89]. This method is more effective with low lignin containing biomass such as agricultural residues, herbaceous crops and hardwoods than on softwood which have a higher lignin content [91]. Furthermore, due to the low severity of the alkaline pre-treatment little sugar decomposition occurs and hemicellulose is retained in the biomass, the method can remove acetyl and various uronic acids which can lower enzyme accessibility [87]. However, use of strong alkali concentrations leads to dissolution, 'peeling' of end-groups, alkaline hydrolysis and decomposition of dissolved polysaccharides [92]. This peeling has advantages but could be at risk of degradation and loss of polysaccharides or carbon in the form of carbon dioxide. To prevent peeling, the temperature is kept low during the extraction process (room temperature or lower) [41]. Research has revealed that applying NaOH at room temperature for 24hr preserves most of the carbohydrates but caused substantial lignin degradation [81]. Research has shown that applying 121 °C autoclaving using NaOH as a pre-treatment on biomass was an impractical temperature, along with the use of pressure of 15 psi, for large scale industrial applications [93]. However, alkaline pre-treatment at room temperature seems to be the best pre-treatment method using caustic materials [94].

The advantages of alkaline pre-treatment are the use of lower temperatures, pressures and residence times when compared to other pre-treatment technologies [95]. Alkali pre-treatments also have lower running costs when using chemicals such as sodium hydroxide, ammonia, peroxide and lime [96]. While, alkaline pre-treatment drawbacks include that these types of pre-treatment have little impact on the solubilisation of cellulose and hemicelluloses [97] and conversion of alkali into irrecoverable salts during the pre-treatment [29].

The efficiency of this process for hydrolysing the organic fraction of MSW has been investigated by using 0.5 – 2M alkali (NaOH, $Ca(OH)_2$, NaOH-urea, Na_2CO_3) at 120-200°C. Use of alkali at these concentrations substantially facilitated saccharification and improved enzymatic hydrolysis [89]. Recent studies have shown that a combined acid/alkaline pre-treatment of lignocellulosic wastes was more efficient than acid or alkaline individually [98].

Alkaline treatment can also be separated into two types on the basis of the alkali employed. These include: Pre-treatment with calcium, sodium and potassium hydroxide

and pre-treatment with ammonia. Ammonia Fiber Explosion (AFEX) treats lignocellulosic biomass (40–50% moisture content) with pure liquid ammonia at mild temperatures (80–100°C) and high pressures (40–50atm) followed by explosive pressure release, pre-treatment helps to disrupt the fibre structure and increases the surface area. The advantage of this process is lower moisture content, lower formation of sugar degradation products and ability for ammonia to reduce lignin inhibitory effect on enzymatic hydrolysis. Disadvantages included costs due to recycling and treatment of chemicals [99].

The mechanism of alkaline hydrolysis depends on solvation and saphonication of intermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin [85]. Solvation and saponification causes the removal of these cross-links and enhances the porosity of the lignocellulosic materials [100]. There are a number of important aspects of alkaline pre-treatment which cause low lignin removal and cellulose swelling; first is that the higher the monomeric hemicellulose fraction, the lower the total recovery of the hemicellulose [101], because the monomeric forms are easily degradable to other volatile compounds for example furfural, which leads to losses of digestible substrate for the ethanol process [102]; secondly, alkali extraction can also cause solubilisation, redistribution and condensation of lignin and modifications in the crystalline state of the cellulose; thirdly, alkaline pre-treatment changes the cellulose stricture to a form a denser and thermodynamically more stable form than the native cellulose [42].

Biological processing

The main biological method for the generation of fermentable sugars is through enzymatic hydrolysis usually after a hydrothermal or chemical pre-treatment. The enzymes are normally produced by microorganisms (fungi and bacteria) and the products of the hydrolysis are usually reducing sugars such as glucose. Cellulase enzymes are mainly used for the hydrolysis of lignocellulosic substrates [103].

The most common microorganisms able to produce hydrolytic enzymes are bacteria belonging to the *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora,and Streptomyces*genera[104], and fungi such as *Sclerotiumrolfsii, Phanerochaetechrysosporium*and species of *Trichoderma, Aspergillus, Schizophyllum and Penicillium (*Sternberg, 1975;Duff and Murray, 1996). In the fungal kingdom, *Trichoderma* has been most extensively studied for cellulase production [105]. However, white rot fungi are thought to be the most efficient for lignin hemicellulose degradation in waste material [106]. White and soft rot fungi attack both cellulose and lignin, the fungi can degrade lignin using enzymes such as peroxidases and laccase, while brown rots mainly degrade cellulose [107].

Enzymes which participate in the hydrolysis of cellulose consist of three major groups: (1) endoglucanase (endo-1,4-glucanohydrolase) which have been shown to degrade low crystalline structures within the cellulose fibre, creating free chain ends; (2) exoglucanase or cellobio hydrolase(1,4-glucan cellobiohydrolase) which remove cellobiose unit from the free chain ends and (3) glucosidases which hydrolyse cellobiose to produce glucose [85]. While, for hydrolysing hemicelluloses, a number of enzymes such as glucuronidase, acetyl esterase, feruloyl esterase, xylanase, xylosidase, galacto mannanase and gluco mannanase are used[108]. The rate and degree of the EH is influenced by: mass transfer resistance, including surface film resistance around cellulose, bulk phase resistance and the resistance through the capillary pores of the cellulose particles [109]. Two major factors contribute to lower hydrolysis rates during EH. Firstly cellulose may be transformed into a less digestible form for enzyme during hydrolysis [110]. Secondly, the soluble products, including glucose and celluobiose, may have a profound inhibiting effect on the action of cellulosic enzymes[111].

The high cost of commercial “cellulose” cocktails is one of the largest obstacles to the economic bio-refinering of biomass which requires large amounts of enzyme [112]. There have been attempts to improve cellulase activity such as direct evaluation and rational design for each cellulase and the reconstitution of designer cellulosome or cellulase mixtures (cocktails) which have a direct activity on the substrate [113]. These improvements include basic studies on fungal physiology and chemistry, cellulase gene regulation and expression, recombinant enzymes, protein engineering of cellulase and development of cellulase enzyme cocktails [114]. The genome of *T. reesei* has revealed that this fungus contains fewer cellulases and hemicellulases than any other sequenced fungi despite being the best known commercial producer of cellulases [115]. Thus other fungal species may harbour more effective enzymes.

Factors affecting the enzymatic hydrolysis of cellulose including substrate condition (pre-treated and unpre-treated), environmental conditions (pH and temperature), and cellulase activity [85].

- **Substrate concentration** is one the main factors which affects the initial rate of enzymatic hydrolysis and yield. Increasing substrate

concentration has resulted in an increase in yield and hydrolysis reaction rates [116]. However, high substrate concentrations can cause substrate inhibition depending on the ratio of total substrate to total enzyme [117]. A 5%w/v substrate concentration achieved the highest rates of hydrolysis, further increase in the substrate concentration decelerated the rate of hydrolysis due to stirring difficulties and reduction in the aqueous mobile phase [118, 119]. Research has revealed that for MSW hydrolysis increased from 27% at 10 g/L to 53% at 50 g/L with a sugar yield of 385 mg sugar/g fibre[120].

- The **structural features** of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin can all affect the susceptibility of cellulosic substrates to cellulases [85]. Pre-treatment of the substrate also influences cellulase enzyme activity (Fig 2.3). The substrate particle size has a significant effect on EH yields, the highest hydrolysis efficiency were observed for the particle size range of 150–300mm. Hydrolysis efficiency increased from 25 to 37% by increasing particle size. This was probably due to the grinding process which may change the surface chemistry or morphology of the fibres making them less accessible to the enzyme. Larger particle sizes above 300 mm resulted in a reduction in sugar yield, this effect can be explained by the longer diffusion of enzyme into the fibre particles suggesting that extensive milling is not needed for the conversion of the organic MSW concentrate [121]**.**
- **Environmental conditions;** increasing incubation temperature has been shown to increase the rate of initial hydrolysis, the maximum hydrolysis rate was observed at 50^{o}C[122]. This result could be attributed to the thermal inactivation of endoglucanase I and cellobiohydrolase I [123]. Studies investigating the effect of reaction time on the sugar yield during EH indicated that by increasing reaction time, sugar yields also increased. However, after 12 hr the hydrolysis rates became constant indicating that some inhibition may occur after that time [124]. The presence of reducing sugars as well as percentage hydrolysis rates decreased with prolonged time after the optimum. This effect may be due to the inhibition of the enzyme action by the accumulated of hydrolysis products [121].
- **Cellulase enzyme**, increasing enzyme dosage increased hydrolysis yields and rates; however, increasing enzyme dosage would also increase the cost of the process. Cellulose enzyme dosage varied from 7 to 33 FPU/g substrate, depending on the type and concentration of substrates [85]. Surfactants can be applied to decrease the irreversible adsorption of cellulase on cellulose but may also be partially responsible for deactivation [125]. Surfactants are also used to block lignin binding to the cellulose and thus enhance enzymatic saccharification of cellulose [126]. There are many surfactants that could be applied such as non-ionic Tween 20, 80 [127], polyoxyethylene glycol [128], Tween 81, Emulgen 147, amphoteric Anhitole 20BS, cationic Q-86W [129], sophorolipid, rhamnolipid, and bacitracin these surfactants are suitable for enhancing cellulose hydrolysis [85, 130]. In addition, the factors affecting activity of Cellulases include enzyme source and the concentration of enzyme. An effective concentration of enzyme for cellulose hydrolysis has been determined to be 10 to 60 FPU/gm of dry cellulose or glucan- glucanase- β- D-glucosidase ratio of 1-75-2IU (International Unit) [131].

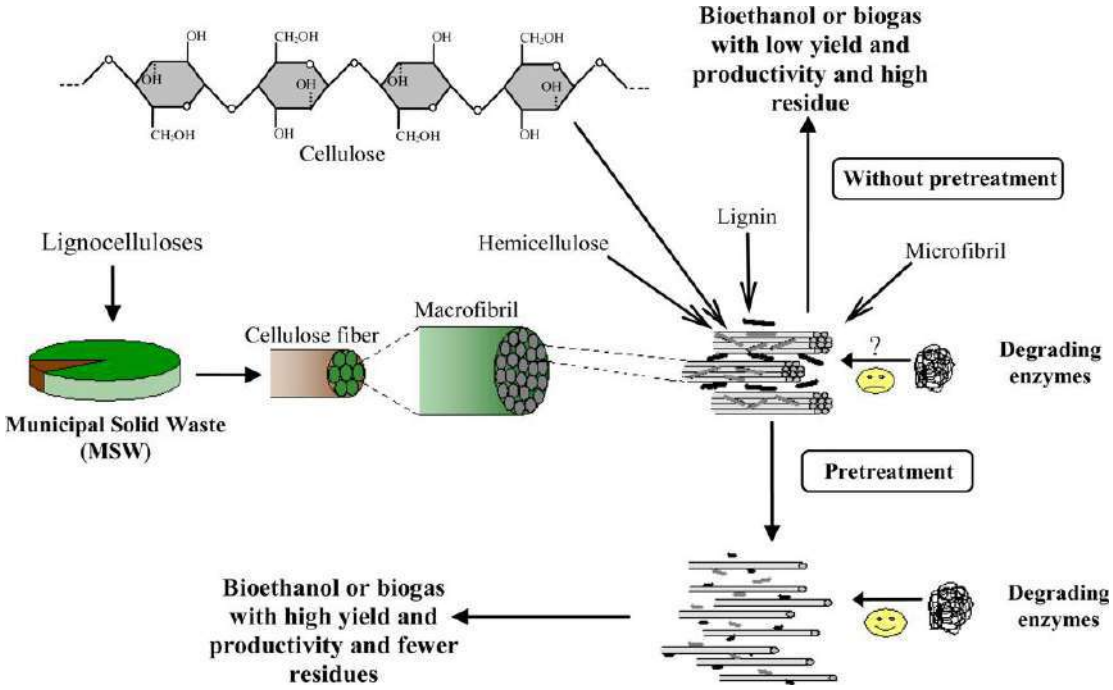

*Fig.2.3 Effect of pre-treatment of MSW on accessibility of degrading enzymes***[28]**

2.3 Biofuel and biochemical production from municipal solid wastes

Fuel production from lignocellulosic biomass, has been well researched, however, MSW has not been given as much attention for energy production when compared with other feed stocks [132]. Fuel production from MSW is a promising strategy for energy needs and effective management of MSW. European legislation aims to minimise landfill use in EU countries, and the amount of biodegradable MSW must be reduced by 65% by 2020 [133]. Studies have shown that up to 82.9 billion litres of waste paper-derived cellulosic ethanol could be produced worldwide, replacing 5.36% of gasoline consumption. The energy independence and security act mandated an increase of 36 billion gallons per year of renewable fuels to be blended into transportation fuels by 2022 [134].

The use of MSW as a biofuel feedstock is dependent on a number of factors which include regional reliability, characteristics of wastes, and compatibility with and efficiency of conversion technologies. Economic factors are cost of collection, transport, and waste conversion. Environmental performance including air and water emissions, greenhouse gases, and finally waste generation; all of these can affect costs and public acceptance [132].

Furthermore, the EPA has defined renewable fuel standards stating that advanced bio-fuels must be derived from feedstock's which meet the definition of renewable biomass. Therefore, MSW must be separated from plastic, rubber, metals and glass [132, 135]. There are many types of biofuel such as ethanol, methanol, bio-diesels which could be used as transportation fuels [134].

2.3.1 Ethanol production

Production of ethanol or ethyl alcohol has existed since the beginning of recorded history [136]. However, since the early 1980's the cost of ethanol production from lignocellulosic biomass was the main concern, at that time the cost was $ 0.95/litre (US $ 3.60/gallon) [137]. However, research has improved ethanol production yield through improved cellulase production, utilisation of a SSF rather than a SMF process, and advances in microorganisms to convert the xylose fraction, as a consequence much better yields and rates have been achieved[138].

The ethanol production process includes using lignocellulosic substrate such as wheat and corn [139]. However, corn is no longer used for ethanol production because of the wide planting for ethanol production which competes for use of arable land and thus threatens national food securities [140]. Lignocellulosic substrates can be used as alternative to corn options which are low in cost and have a high polysaccharide content [141]. Nowadays food wastes can be utilised as substrates for ethanol production, research carried out has shown that sweet potato can be converted into ethanol with a 80.23% yield [142].

A few studies has been applied for bioethanol production from MSW,[19] showed that using pre-treatment with dilute sulphuric acid followed by steam explosion did

increase the rate at which the maximum yield of glucose was formed. However, this pre-treatment did not give high yields for newspaper wastes.

Another study used selected biodegradable MSWfractions; these fractions were subjected to fifteen different pre-hydrolysis treatments to obtain the highest glucose yield for bio-ethanol production. Glucose yields were compared using a factorial experimental design. The highest glucose yield (72.80%) was obtained with a pre-hydrolysis treatment consisting of H_2SO_4 at 1% concentration, followed by steam treatment at 121°C, and enzymatic hydrolysis at 60 FPU/g substrate [143].

A study by Yan *et al*. (2012) using enzymatically hydrolysed food wastes showed that batch and fed batch hydrolysates, which contained reducing sugar concentrations of 131.41 and 194.43 g/L respectively, produced 62.93 and 90.72 g/L, ethanol following fermentation using *Saccharomyces cerevisiae* H058, for 48 hr.

There are three major stages involved in the conversion of lignocellulose to ethanol - pre-treatment, enzymatic hydrolysis and fermentation. This is followed by distillation to extract pure ethanol. The steps, or technologies, required for ethanol production are shown in Fig (2.4).

(1) Pre-treatment

Pre-treatments using physical, physicochemical, chemical and biological methods as mentionedpreviously,are an important step to make cellulose more accessible in the hydrolysis step. However, this is a costly step, accounting for approximately 33% of the total cost [144].

(2) Enzymatic hydrolysis

EH is the process used to convert polysaccharides into simple sugars, which can be fermented by bacteria or yeast [145].

In the second stage, the conversion of cellulose and hemicellulose can be expressed by the reaction of glucan (for hexoses) and xylan (for pentose) with water:

$$(C_6H_{10}O_5)n + nH_2O \rightarrow n\ C_6H_{12}O_6 \quad \text{.....(1)}$$

$$(C_5H_8O_4)n + nH_2O \rightarrow n\ C_5H_{10}O_5 \quad \text{.....(2)}$$

The maximum theoretical yield of hexoses and pentoses is 1.136kg and 1.111kg per kg of glucan and xylan, respectively [146].

(3) Fermentation

The fermentation reaction by yeast and bacteria of simple sugars produces bio-fuels such as ethanol or butanol, CO_2 is also produced during the fermentation process. The simplified reaction equation is: [146]

The conversion reaction for hexoses (C6) and pentose (C5) are as follows:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2CO_2 \quad \text{..... (3)}$$

$$3C_6H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5CO_2 \quad \text{..... (4)}$$

The theoretical maximum yield of ethanol from hexoses and pentose is 0.511kg ethanol and 0.489 kg CO_2 per kg sugar, respectively [146].

Generally yeast such as *S. cerevisiae* are used for ethanol production, however, this yeast cannot metabolise xylose efficiently[147, 148].However, many bacteria and yeast are able to ferment xylose and other pentose sugars either naturally or following genetic manipulation[149, 150].

Yeasts when utilised for ethanol production are required to be capable of fermenting all of the sugars present with high ethanol yields. Wild-type *S. cerevisiae* strains are unable to ferment pentose sugars; its capability for xylose utilization has been improved by intensive recent research. During the last fifteen years, research has been focused on finding xylose fermenting microorganisms and understanding the principles behind the utilisation of xylose [146]. *S. cerevisiae* has desirable characteristics such as efficient anaerobic sugar metabolism, toleration of inhibitory industrial substrates better than other microorganisms and ferments hexoses abundantly present in lignocellulosic hydrolysates, such as glucose, mannose and galactose with high yield and productivity [85].

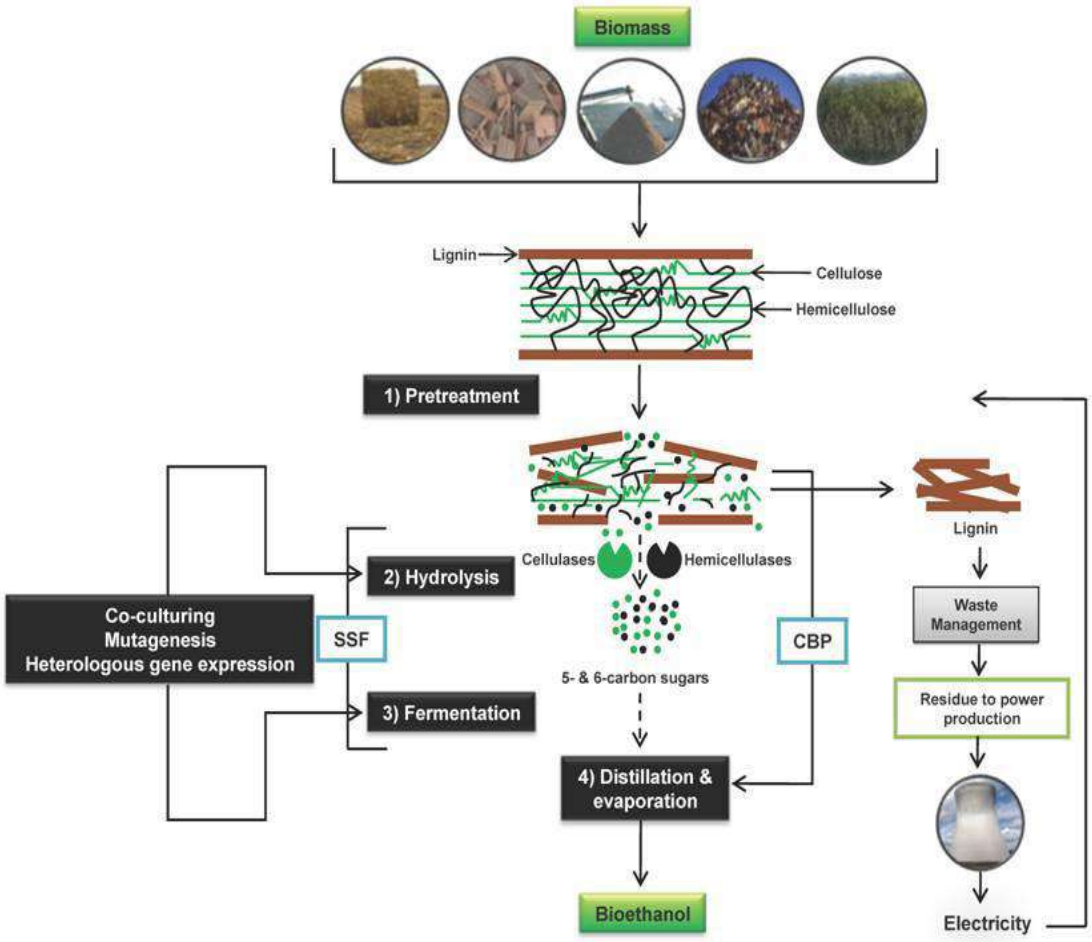

*Fig.2.4 Schematic for the conversion of lignocellulosic biomass to ethanol***[151]**

2.3.2 Butanol production

1-Butanol (butyl alcohol or n-butanol) is a four carbon straight chained alcohol with a molecular formula of C_4H_9OH (MW 74.12) and boiling point of 118°C [152]. Butanol is a good source of biofuel [153], because it has low vapour pressure, can be blended with either gasoline or diesel at any fraction [154]. Butanol has some advantages over ethanol as a fuel substitute, it has an energy content that is similar to gasoline, lower vapour pressure compared to ethanol and is safer during transport and when used in car engines[155].Butanol can also be used as an important chemical precursor for paints, polymers, plastics, solvents, plasticizers, butylamines, amino resins, butyl acetates production, et[152, 156]. Therefore, bio-butanol has the potential to substitute for both ethanol and bio-diesel in the biofuel market and is estimated to be worth $247 billion by 2020 [152].

For butanol chemical production of Oxo synthesis, Reppe synthesis, and crotonaldehyde hydrogenation are the three most important processes, most of these process rely on petroleum, however, butanol can be produced from biomass [157]. Butanol is currently manufactured from petroleum feedstock (Oxo process). While, Bio-butanol is produced via the Acetone Butanol Ethanol (ABE) fermentation process using renewable resources (biomass) and *Clostridium acetobutylicum* or *Clostridium beijerinckii*in anaerobic conditions) [154]. Conventional butanol fermentation is carried out by microorganisms in a two-stage batch process: an initial acidogenic stage followed by a solventogenic stage. This fermentation process is known as ABE production [158].

The microorganisms used for butanol, acetone, ethanol are production usually formed by *Clostridia* bacteria, these bacteria can degrade a number of toxic chemicals by producing chiral products which are difficult to make by chemical synthesis[159]

Currently butanol is produced from the fermentation of corn, cassava or molasses as substrates[160, 161]. Different types of biomass such as wheat straw[162, 163]rice straw [164], barley straw [165], corn stover[166], corn cob and fibres [167], palm kernel cake [168], cassava starch [169], pinewood and timothy grass [162], switch grass [170], have been used as substrates for ABE fermentation by numerous *Clostridium* strains [155]. MSW

has also been applied to reduce the cost for the biofuel market and has been shown to be more sustainable offering a lower carbon footprint and reduced GHG emissions [152]. Some studies have applied domestic organic waste (DOW) as a substrate for butanol production, using steam explosion and enzymatic hydrolysis for the washed and dried DOW produce using *Clostridium acetobutylicum* DSM 1731 produced 1.5 and *C. beijerinckii* B-592 0.9 g/L ABE and *Clostridium* LMD 84.48 1.9 g/L Isopropanol, Butanol, Ethanol (IBE) [171].

ABE fermentation is also used with domestic organic waste and *C. acetobutylocum* in a batch fermentation [171].Utilization of such waste materials improves the economy of butanol production [172].

2.3.3 Organic acid production from municipal solid waste

Organic acids are promising bio-energy products that could be produced using renewable carbon sources and microorganisms but are not currently produced at a large-scale processes. However, citric, lactic and succinic acids are three products at different stages of industrial development [173].

These acids are produced naturally by microorganisms, or are at least natural intermediates in major metabolic pathways. These acids are important for example succinic, fumaric and malic acid could replace the petroleum-derived commodity chemical maleic anhydride [174](Fig2.5).

To produce organic acids, various cheap substrates has been selected such as red lentil flour in India [175], kitchen waste in Japan [176], barley hydrolysates in the EU [177] and oat [178] or liquefied corn starch from cassava bagasse [179].

- **Citric acid**

Citric acid is widely use in the food and pharmaceutical industries. 70% of the food industry is dependent on citric acid followed by about 12% for the pharmaceutical industry and 18% for other applications [180]. Citric acid can be obtained by chemical synthesis by the filamentous fungus *Aspergillusniger*. In addition to fungi, yeast have been applied and developed as a microbial cell factory for citric acid [181]. *Yarrowialipolytica* is also used for the production of citric acid from carbon sources, such as glucose and sucrose [182].

Various agro-industrial residues such as apple pomace, coffee husk, wheat straw, pineapple waste, mixed fruit, maosmi waste, cassava bagasse, banana, sugar beet cosset and kiwi fruit peel have been investigated for their potential to be used as substrates [183].

Nowadays production of citric acid is approximately 1.6 million tons. There are many parameters that help to get highly efficient biotechnological production of citric acid such as “high substrate concentration, low and finite content of nitrogen and certain trace metals, thorough maintenance of high dissolved oxygen, and low pH”. Currently the production of citric acid is approximately 1.6 million tons (t) [184].

- **Lactic acid**

Lactic acid has been widely applied in the food, pharmaceutical, leather and textile industries and as a chemical feedstock. Currently, lactic acid is used as starting material to produce biodegradable polymers which are then used in medical, industrial and consumer products[185, 186]. The acid is produced by *Rhizopousoryzae* using SSF with sugarcane bagasse [187] or by *Lactobacillus paracasei* in solid-state conditions using sweet sorghum [188].

- **Succinic acid**

Currently, the succinic acid market is small at around 16,000 tons per year; this acid could replace petroleum derived maleic anhydride, which has a market volume of 213,000 tons/year. Deriving succinic acid from petroleum causes environmental pollution [189]. Microorganisms like *Escherichia coli* and filamentous fungi, including *Penicillium simplicissimum*, have been shown to naturally accumulate succinic acid [190].

- **Gluconic acid**

Gluconic acid is used widely in the food, pharmaceutical, cement, textile and chemical industries and is in high demand at 50,000–60,000 tons/annum. Gluconic acid is an oxidative product of the glucose industries[191, 192]. Solid state fermentation (SSF) and Sub merged Fermentation (SMF) have been used to produce gluconic acid using *A. niger*[193]. Various substrates have been used for gluconic acid production such as sugarcane molasses which have a high economic benefits in-terms of cost, by using SMF, many studies have applied SSF for gluconic production to reduce the cost [194].

- **Oxalic acid**

Oxalic acid and their salts can be used as a bleaching agent, in detergent formulation and as a metal polisher because of its capacity for reducing iron and other metals compounds [195]. Oxalic acid is also used as a mordant in dyeing processes. There are two ways to produce oxalic acid by either chemical or biotechnological processes, a chemical method uses formic acid salts (heating sodium

formate and treating the resulting oxides with sulphuric acid) or by carbohydrate oxidation by nitric acid [196]. Oxalic acid can be produced using biotechnological methods which include using microorganisms such as *Cyanobacteriae*[197], brown rot fungi [198] and other fungi, such as *Penicillium*[199], these organisms secrete oxalic acid at low concentrations. *A. niger* produces not only oxalic but also citric and gluconic acids according to the operating conditions [200]. Oxalic acid production efficiency depends on factors such as C- and N-source and the initial medium pH as well as the culture/broth pH during fermentation[201].

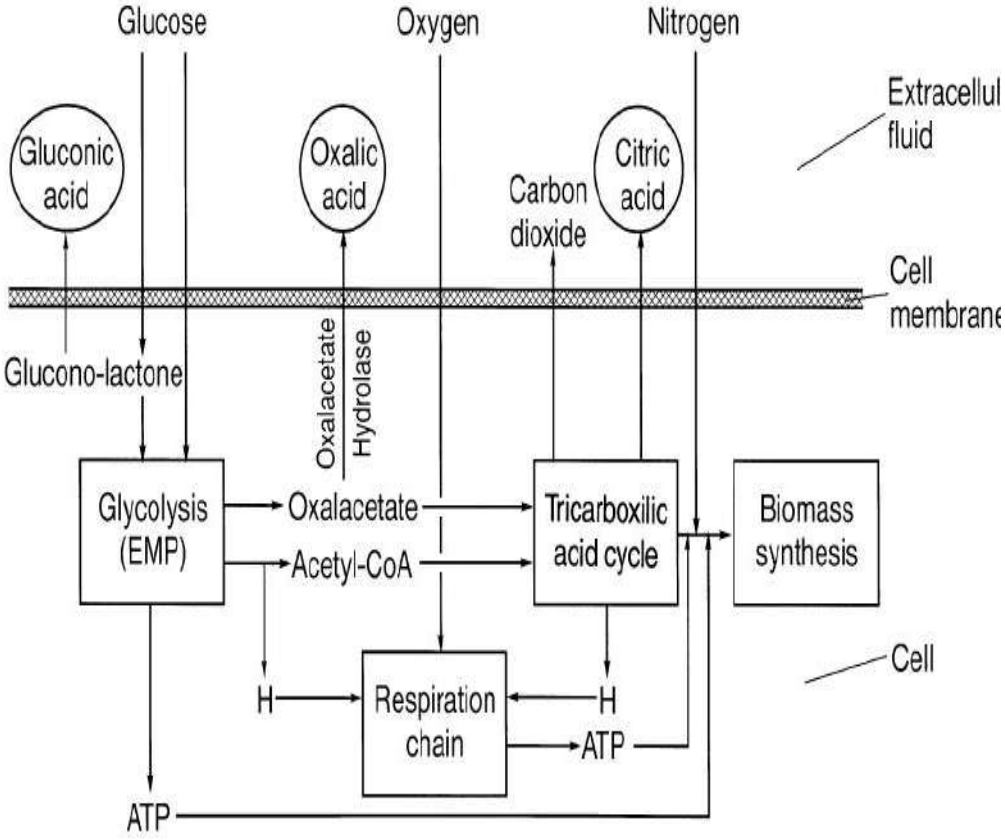

*Fig.2.5 Metabolic pathway for citric, gluconic and oxalic acid synthesis in A. niger***[196]**

2.4 Enzyme Production

2.4.1 Use of microorganisms for enzyme production from biomass and MSW

Solid state fermentation (SSF) is currently used for the production of various commercial enzymes [132]. These include those enzymes involved in the degradation of biomass. The substrates used in SSF can be classified into two categories: inert materials, which only act as an attachment for the microorganism and non-inert materials, which not only function as an attachment but also supply nutrients to the microorganism [202]. Several parameters need to be taken into consideration to insure a successful SSF process these include environmental parameters (temperature, pH, water content and activity) and the carbon source (biomass, substrate concentration, CO_2) [18].

pH plays an important role for cellulase production and the impact of initial pH of the culture medium has been extensively investigated. Research has revealed that the maximum cellulase activity from corn stover was obtained at pH 2 [203], at optimal temperature the optimal pH is 3-6 using fungi [204]. For SSF, it is difficult to monitor the pH and is normally not controlled during the SSF process and can only be adjusted at the beginning of the process [205]. It has been reported that in the first 4 days of SSF the initial pH drops and then increases after 8 days using rice straw [206]. The initial decrease in pH is due to the formation of organic acids and consumption of ammonium salt in the fermentation media[207].

Temperature, has an obvious effect on germination of spores [204]. The optimal temperature for *A. niger* growth is room temperature because this is similar to the natural habitat of the fungi, which is classified as a mesophilic microorganism [208].

Moisture is a crucial factor which affects metabolite production in SSF, because when the moisture level is low the solubility of nutrients decreases[209, 210]. However, high levels of water in the SSF media means that substrate particles will be surrounded by a thick layer of water, these particles stick together and limit the diffusion of air between the particles and the immediate surroundings [211]. The presence of water helps to swell the substrate and facilitates absorption of the nutrients from the substrates for growth and metabolic activities [212]. The nature of the substrate, porosity, specific surface area the requirements of microorganisms and the type of end product determines the optimal moisture conditions [213].

Inoculation size, for SSF inoculums preparation include spore suspension, mycelia disc, and mycelium suspension and pre-inoculated substrate [205]. Initially spores attach to the outer surface of the substrate particles and grow slowly, multiplying and penetrating into the substrate [214]. Optimal spore suspension concentration used in research is approximately 10^6 spore/mL[206, 215].

Incubation period has a significant role but maybe affected by several factors, such as the presence of different ratios of amorphous to crystalline cellulose [216]. The first signs of fungal growth were reported on day two of SSF for cellulase enzyme production and after 7 to 11 days the fungus completely colonised the substrate depending on the type of substrate used[217, 218]. During the colonisation phase of fungal growth, extracellulase enzyme was produced to degrade the lignocellulosic substrate into small particles, which helped fungal growth as a nutrient source [219].

Supplements - to increase cellulase activities some type of supplements such as carbon and nitrogen sources can be added to the substrate [220]. Generally cellulose in a lignocellulosic substrate acts as an essential carbon source, also fungal and cellulase production can be stimulated by nitrogen source, peptone can be used as a nitrogen source and is able to increase enzyme production, it's essential to have the proper combination of nitrogen source, lignocellulosic substrate and fungal strain for maximizing cellulase production [221]. Phosphorus, trace elements and other minerals can also be supplemented into the SSF media and play important roles, phosphorus helps the formation of phospholipid bilayers in the fungal cell membrane [222]. Addition of a surfactant such as Tween 80 and triton X-100 to the fermentation medium can help to improve the permeability of fungal cell membrane thus allowing the secretion of cellulase in a more rapid manner [223]. Some trace elements such as Zn^{2+}, Ni^{2+}, $Mn^{2+}$and Co^{2+}which serve as cofactors, may enhance cellulase enzyme production [224]. The presence of heavy metals could also interfere with energy supplying system for cellulase production for example cellulase of *P. chrysosporium* in liquid medium was inhibited in the presence of 50–150 ppm Cd^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}. At 150–300 ppm Mn^{2+}or 300 ppm Cd^{2+} or Co^{2+}, no cellulase activity was detected[225, 226].

Particle size, the surface area plays an important role for microbial attachment, mass transfer of various nutrients and substrates and subsequent growth and product formation[227].

Type of lignocellulosic substrate, selecting a substrate that is able to support fungal growth, stimulates cellulase production and contains sufficient nutrients is particularly important. Selecting a substrate that enables the anchorage of fungi during fungal growth is also an important criterion before applying it for SSF [228]. In addition, there are many other approaches being taken to enhance cellulase production, such as genetic modification (mutagenesis, heterologous expression) of fungal strains and co-culture of different fungal strains [205].

2.4.2 Application of SSF using various biomass

SSF has been employed for the production of antibiotics, surfactants, biocides and enzymes [202], these products can be produced using bacteria, yeast and fungi. These microorganisms are capable of growth on solid substrates, among these microorganisms filamentous fungi have been commonly employed due to their physiological capabilities and hyphal mode of growth under conditions of low moisture [229]. Potential applications of SSF included:

I. Production of commercial products.

Industrial residues can be converted into valuable products, for example coffee (pulps and husks), soybeans, cassava husk and bagasse, sugarcane bagasse, sugar beet pulp, fruit wastes, palm tree wastes bio-converted into single cell protein, organic acids like citric and lactic acids, amino acids, pigments, antibiotics, mushrooms, bio-pesticides, gibberellic acid, flavour and aroma compounds[229].

II. Environmental control

The SSF process helps in the biodegradation of hazardous compounds, use of SSF has shown promise for the biological detoxification of industrial wastes and insecticides and for pest control in crops [202].

III. Food industry products

SSF has been used in the production of food additives or flavouring compounds [230], these compounds are produced via chemical synthesis or by extraction from natural materials [231]. Several microorganisms have the ability to produce aroma compounds from agro-industrial

wastes [232]. Some aroma compounds such as monoterpene alcohols and isoamyl acetate have been produced by *Kluyveromycesmarxianus* from cassava bagasse [233].

IV. Enzyme production

Several enzymes can be produce by SSF using lignocellulosic wastes [234], recent studies confirmed that SSF is the best system for producing enzymes and better than SMF with regards to yields obtained[235, 236]. Generally, the most common industrial enzymes produced using SSF are proteases, cellulase, ligninases, xylanases, pectinases, amylases, glucoamylases; also production of inulinases, phytases, tannases, phenolic acid esterases, microbial rennets, aryl-alcohol oxidases, oligosaccharide oxidases, tannin acyl hydrolase, a -L-arabinofuranosidase[202].

The most common enzyme produced is cellulase enzyme **"**Cellulases are a complex enzyme system, comprising endo-1,4-b -D-glucanase (EC-3.2.1.4), exo-1,4-b-glucanase (exocellobiohydrolase, EC-3.2.1.91) and b-D-glucosidase (b-D-glucoside glucanhydrolase, EC-3.2.1.21)"[202]. Cellulases are one of the largest groups in the structural classification of glycosyl hydrolyses, this classification is based on variability of catalytic domains and does not consider variability in cellulose binding domains[235, 236]. Cellulase is recorded as the third largest industrially produced enzyme, and is applied widely in cotton processing; paper recycling, juice extraction, detergents and as an animal feed additives. Cellulase is also commercially produced for saccharification of biomass[236, 237].

The enzyme is produced by various microorganisms (fungi and bacteria),including *Aspergillus* fungi [238]. Furthermore, the most investigated and genetically improved for fungi enzyme production are the *Trichoderma spp.*[239]. Generally, *Aspergillus* and *Trichoderma spp*. are well known efficient producers of cellulases [240] for example *T. reesei* produces 2 Cellobiohydrolase (CBH), 8(endo-B-1-4-glucanase (EG) and 7 B- glucosidase [241]. Commercially most enzymes are produced from these two strains of soft rot fungi, but *T. reesei* is not capable of producing substantial amounts of B-glucosidase, whilst *A. niger* produces a cellulose system lacking endo and exoglucanase[223].

In addition, the most important cellulolytic microbes which are able to produce cellulase obtain their energy primarily from carbohydrates and are unable to use lipids or proteins [235]. Fungi possess the ability to secret large amounts of extracellular protein; such strains are most suited for production of high levels of extracellular cellulase. The most commonly studied cellulolytic organisms is *T. reesei*[242].

SSF is gaining interest as a cost effective technology for cellulase enzyme production and bioconversion of lignocellulosic biomass using cellulolytic microorganisms. Comparing liquid culture with solid state culture for cellulase enzyme production, SSF has been shown to closely resemble the natural habitat of filamentous fungi, also enzyme titre produced from SSF is superior compared to the titre produced via SMF[243].

Advantages of SSF over SMF: SSF is a process which requires smaller amounts of water and therefore the cost of the process can be greatly reduced [244]; SSF has been shown to produce higher concentrations of enzymes; higher fermentation productivity and has a lower demand on the sterility of the equipment[245].Finally, the crude enzyme extracted from SSF can be applied directly to hydrolyse the lignocellulosic substrate [205]. Studies have reported a higher yield of cellulase from *T. reesei* using SSF compared with SMF processes [246]. Furthermore, some studies have proposed that SSF application could be a better technology for commercial production of cellulase with low cost, and by using naturally available cellulose sources [202].

2.4.3 Mechanisms of SSF for cellulase enzyme production

There has been extensive research on cellulase enzyme production [247]. For example the rate-limiting step for crystalline cellulose degradation has yet to be determined. It is not clear which segment of the cellulose fibrils cellulase binds to. A further unknown is how cellulosomes are able to efficiently catalyse the hydrolysis of cellulose and how free cellulose binding modules stimulate cellulase hydrolysis [248]. Finally, how mixtures of cellulases hydrolyse both crystalline and amorphous regions in bacterial cellulose, while most individual enzymes only seem to degrade amorphous regions [249].

Besides wheat straw, other cheap materials, such as banana peel, rice straw, corn cob residue, rice husk, wheat straw, banana fruit stalk, and coconut coir pith are all being used for cellulase production[250, 251] (Table 2.1).

Table.2.1 Applying various Substrates and Microorganisms for cellulase enzyme production

Substrate	Microorganism	Yield	Reference
Rice barn and corn straw	*Trichoderma reesei*	Cellulase18.5 IU/mL	[252]
Egg shell waste	*Neurosporacrassa*	Cellulase 2.30U/mL	[253]
Water hyacinth	*Trichoderma reesei SEMCC-3.217*	Cellulase 13.4 FPIU/g dry solid	[254]
Xylose industry	*Trichoderma reesei ZU-02.*	Cellulase (158 IFPU/g)	[255]
Vinegar industry	*Trichoderma koningii AS3.4262*	Cellulase 6.90 IU/g of substrate dry matter (SDM)	[256]
Wheat bran	*Trichoderma reesei*	Cellulase 2.63 U/ mL	[257]
Sugar cane bagasse	*Trichoderma reesei NEEL 11460*	Cellulase 154.58U/gds	[258]
Sweet potato	*Bacillus sp*	Amylase and cellulase 28 U/mL	[259]
Oil palm in the form of empty fruit bunches	*Trichoderma harzianum T2008*	Cellulase 8.2 U/gm	[260]
Saw dust and bagass	*Aspergillus niger*	Cellulase Sawdust gave the best result with an enzyme activity value of 0.0846 IU/mL while bagasse gave 0.0682 IU/mL	[261]
Rice bran	*Trichoderma reeseii QM9414 and T. reesei MCG77*	Cellulases 1.1635 U/g	[262]
Rice straw	*Acremoniumcellulolyticus*	Cellulases and hemicellulases DBMc, 10.8 FPU/mL and WDMc, 10.4 FPU/mL	[263]
Cocoa (Theobroma cacao) meal	*Aspergellus niger*	Cellulase 14.18 U/mL and xylanase11.86 U/mL	[264]
Banana waste	*Bacillus subtilis (CBTK 106),*	CellulaeThe optimal ®lter paper activity (FP Ase) of 2.8 IUgdsÿ1, CMCase activity of 9.6 IUgdsÿ1 and cellobiase activity of 4.5 IUgdsÿ	[265]

2.5 Composting and fertiliser

2.5.1 Compost and fertiliser production from municipal solid waste

Waste contain various levels of metals, some of them are discharged directly or indirectly in to the environment, which can cause serious environmental pollution, and threaten life [266, 267]. These metals are classified into the following three categories: toxic metals (such as Hg, Cr^{6+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, As^{3-}, Co^{2+},Sn, etc.), precious metals (such as Pd^{2+}, Pt, Ag^{+}, Au, Ru etc.) and radionuclides (such as U, Th, Ra, Am, etc.) [266].

In recent years, leaching which is used to remove metals from aqueous solution has been carried out using methods such as chemical precipitation, ion exchange, electrochemical treatment membrane technologies, adsorption on activated carbon etc[268].

The major advantages of bio-sorption over other methods include low cost, high efficiency, minimisation of chemical and biological sludge, no additional nutrient requirement, regeneration of bio-sorbent, and possibility of metal recovery [269]. The bioleaching process has many advantages: economically, the process is cheap and simple to operate, has lower energy requirement; environmentally, the process is environmentally friendly because there are no by-products e.g. gaseous pollutants are produced in bio-hydrometallurgy [270]. In addition, bioleaching has potential for the environmental clean-up of mining sites, treatment of mineral industrial waste products, detoxification of sewage sludge and for the remediation of soils and sediments contaminated with heavy metals [271, 272].

Microbial bioleaching is based on the natural ability of microorganisms to transform solid compounds to a soluble and extractable form. This may involve enzymatic oxidation or reduction of the solid compound, or an attack on the solid compound by metabolic products [273].

Bioleaching has been defined as the interaction between metals and microorganisms, which leads to solubilisation of metals in a solid form. Additionally, the term "bio-oxidation" is also used [274]. In addition, leaching can be direct (i.e., physical contact between microorganisms and solid material) or indirect (e.g., bacterial oxidation of Fe^{2+} to Fe^{3+} which catalyses metal solubilisation as an electron carrier) [275].

Various microorganisms can be applied for bioleaching processes such as autotrophic bacteria, heterotrophic bacteria, and fungi. Fungi belonging to the *Aspergillus* and *Penicillium*genus have been the most extensively studied [271],marine algae (eg*Sargassumnatans)*, yeast (eg. *S. cerevisiae*) have also been studied. Fungi are able to solubilise metal compounds by excreting acid, mainly in the form of organic acids, using heterotrophic fungi results in a faster leaching process and with a shorter lag phase; for example by using MSW fly ash as the substrate *Aspergillus thiooxidans* required 1-3 months, while *A. niger* only requires 2-3 weeks to complete the leaching process [276]. Addition of organic acids helps to increase the solubility of metal ions at non-acidic pH values by chelating, in addition, complexation between metal ions and organic acid anions may reduce their toxicity [271].

Fungi can withstand a much wider pH range, typically from 2 to 7, media composition and leaching environment[195, 271].

The organic acids are produced by fungi in complexes with metal ions and enhance metal solubilisation, these complexes help to reduce the toxicity of heavy metal ions to the fungi, and thus the fungi are fairly resistant to heavy metals [195]. Microorganisms are able to mobilize these metals by various reactions[277].

Currently, *S. cerevisiae* is also used for heavy metal bioremediation, yeast bioleaching strains are affected by many factors such as pH, redox potential, presence of anions and soluble organic compounds [278]. *S. cerevisiae* has advantages for bioremediation as the yeast is a mediocre bio-sorbent, easily cultivated at large scale, has a high yield of biomass, can be easily manipulated genetically and the complete genomic sequence is available [279]. In addition, research has shown that *S. cerevisiae* has the ability to remove toxic metals by accumulating metals in an external layer of the cell wall [280].

Due to ash being rich with nitrogen, phosphorous and potassium which are the main nutrients for plant growth, these MSW leachates could be used as a replacement for commercial fertilisers. In addition, application of lime or alkali substance can reduce soil acidity. However, the heavy metals present in MSW ash are toxic for plants and animals, removing metals makes it more applicable in the agriculture field [281]. One study has shown that MSW fly ash, bottom ash and combined ash can influence plant growth in a positive manner. Growth of alfalfa and Swiss chard in ash-amended soils was similar to that in soils amended with phosphorous and potassium fertiliser, indicating that MSW ash can supply essential nutrients for plant growth[6].

2.5.2 Mechanisms of Bioleaching

In the environment, heavy metals are present at low concentrations in the soil; however, these metals can be toxic at higher level such as zinc and copper, while others like aluminium and lead are only known for their toxicity. Soil acidity helps to dissolve metal containing minerals and increases uptake by plants, which causes metal toxicity as the plasma membrane of root cells is often damaged by exposure to toxic metals, resulting in leakage of cellular solutes. However, there are some plants called edaphic ecotypes which can tolerate the presence of heavy metals in the soil [282].

Nowadays, there are many causes increasing the concentration of heavy metals in the environment [283]. The composting process has been defined as the biological decomposition of organic matter by adverse population of microbes under controlled aerobic condition to form stable humus –like end products [284].

Fungi can tolerate metals using two mechanisms: firstly, extracellular (chelation and cell-wall binding) sequestration, this step avoids metal entry into the cell

[285]. The second mechanism is the intracellular physical sequestration of metal either by binding to proteins or other ligands preventing damage to cellular targets. In this mechanism metal transport proteins may be involved in metal tolerance, either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into the vascular compartment[286, 287](Fig 2.6).

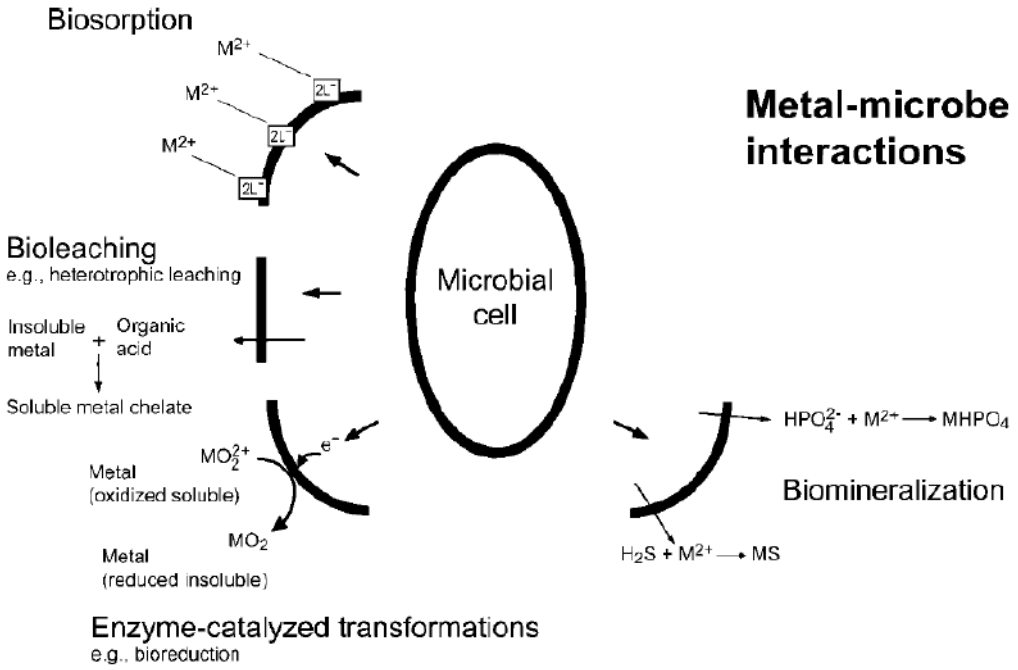

Fig.2.6 Mechanism of metal-microbe interactions that can be harnessed for bioremediation application **[288].**

The ability of microorganisms (bacteria and fungi) to mobilize and leach metals from solid materials is based mainly on three principles [277]:

I. Redoxolysis (oxidation and reduction reactions)

Divided into direct and indirect mechanisms: **direct mechanism**: metals are solubilised through enzymatic reaction, through physical contact between the leaching materials and microorganisms. Leaching a metal from a solid structure may occur through oxidation or reduction reactions. This involves the transfer of electrons either from the solid structure to an electron acceptor like O_2 called oxidation or the injection of electrons into the solid structure from an electron donor like H_2termed reduction [289].

Direct bacterial leaching can be described according to the following reaction:

$$MeS + 2\,O_2 \xrightarrow{\text{bacteria}} MeSO_4$$

In the above, MeS is the metal sulphide [290], direct leaching benefits the autotrophs, because they conserve energy during the process [291].

Indirect redox mechanism causes oxidation of ions originating from the microbial oxidation of ferrous iron (Fe^{2+}) compounds, which helps to dissolve metals from the solid chemically. Ferric iron is an oxidising agent [277]. Redoxolysis of fungal bioleaching is a reduction of ferric iron and manganese, mediated by oxalic acid in an acidic environment [195]. Indirect leaching of metal sulphides can be described by the following reactions [273]:

$$2\,Fe^{2+} + 2\,H^{+} + \tfrac{1}{2}\,O_2 \xrightarrow{\text{bacteria}} 2\,Fe^{3+} + H_2O$$

$$2\,Fe^{3+} + MeS \xrightarrow{\text{abiological}} Me^{2+} + S + 2\,Fe^{2+}$$

$$S + H_2O + 3/2\,O_2 \xrightarrow{\text{bacteria}} H_2SO_4$$

II. Acidolysis (the formation of organic or inorganic acids)

In acidolysis, organic acids are formed by bacterial metabolism resulting in organic acidolysis, complex and chelate formation (e.g. production of citric acid or gluconic acid by *A. niger* or *P. simplicissimum*, and sulphuric acid by *A. ferrooxidans* and *A. thiooxidans*) [292]. The acidolysis mechanism is solubilization of heavy metals by

bio-produced acids, this step plays the most important role in bioleaching process [293].

Mineral solubilisation occurs simultaneously in the presence of ligands under acidic conditions. A kinetic model of the coordination chemistry of mineral solubilisation has been developed which explains the dissolution of oxides by protonation of the mineral surface and the surface concentration of suitable complex forming such as oxalate, malonate, citrate and succinate [292]. The protons and the oxygen combine with water and the metal is therefore detached from the surface [195].

$$MeO + 2\,H^{+} \longrightarrow Me^{2+} + H_2O$$

In the above, MeO is the metal oxide.

Protons are obtained from the acids produced, and the maximum amount available determines the amount of metal oxides solubilized. This process is usually fast and it is the most important mechanism for fungal bioleaching. In the above, MeO is the metal oxide [294].

III. Complexolysis (the excretion of complexing agents)

The third mechanism including extraction of metals by complexing agents, to form soluble metal complexes, organic acids can leach metals through complexation. Complexolysis is a slower mechanism when compared with acidolysis, metal dissolution depends on the complexing capacity of molecules and bonds in the solid particles, so if the bond between metal ions and ligands are stronger than the lattice bonds between metal ions with solid particles, the metal will be successfully leached out from the solid particles [291]. Additionally, the complexation of heavy metals can reduce metal toxicity to the fungi when high concentrations of metals are present [271].

IV. Bioaccumulation

Bioaccumulation is another important mechanism or process in fungal bioleaching. in this process the mycelium functions as a "sink" for the metal ions and causes continuous solubilisation of the metals by the accumulation of metal ions from the leaching solution through active metabolic reactions and passive adsorption, this continuous solubilisation upsets the equilibrium between the solid and dissolved metal. In addition, the fungal cell wall contains many different functional groups (e.g. hydroxyl, amine, carboxyl, phosphate and sulphydryl groups), which are able to bind metal ions to a greater or lesser extent [295].

2.5.3 Factors Influencing Bioleaching

The effectiveness of leaching depends largely on the efficiency of the microorganisms, chemical and mineralogical composition of the material to be leached and leaching conditions. The maximum metal extraction can be achieved only when optimum conditions are employed [296].

Nutrient Culture Media - Effective microbial growth, biosynthesis of new cells and metabolism requires nutrients in order obtain to get maximal growth some selective nutrients help in the production of the necessary metabolites for bioleaching. The presence of ammonium, phosphate and magnesium salts have been shown to increase growth rates; inorganic iron and sulphur compounds are required for chemo-litho-autotrophs [296]. For leaching metals some nutrients help to increase the production of organic acids and scavenge metals [297]. Research has revealed that potassium deficiency increased oxalic acid production significantly by tree seedlings colonised by the fungus *Paxillus involutus*, while Mg^{2+} deficiency increased oxalate production in both mycorrhizal and non-mycorrhizal tree seedlings in the same experiment [298]. Furthermore, carbon source plays an important role in the determination of quality and quantity of organic acid production [186].

Microbial type: There's a diverse range of mechanisms that microbes can adopt in bioleaching processes because of difference in their metabolic activities. These differences can be intra- or inter-species, depending on other factors such as exposure to high levels of heavy metals. For example, *Aspergillus* and*Penicillium* have mutants that can withstand heavy metals and a genetic adaptation (mutation) [186]. Aerobic and anaerobic microorganisms are both involved in many biohydrometallurgical processes; these microorganisms require adequate oxygen or CO_2 to get their optimum growth and activity. Aeration, shaking or stirring are some of the common methods employed in the laboratory to supply oxygen or CO_2 to the microbes because of insufficient oxygen or CO_2 cause slow microbial growth as a result there is a decrease in the metals leaching rate [271].

pH and temperature: Optimal microorganism growth is pH dependent as is solubilisation of metals. It is known that a low pH is the most favourable condition for metal solubilisation[299].Temperature plays a role in a bioleaching process, an optimal temperature should be maintained according to the optimal microbial growth condition. Mesophilic microbes grow at temperatures of

28-35°C, while thermopiles grow at temperatures above 50°C [271, 296].

Metal Resistance of Microorganisms: Leaching metals from the substrate is accompanied by an increase in metal concentration in the leachate. Generally heavy metals exhibit toxic effects due to four factors: (1) the blocking of functional groups of biologically important molecules, (ii) the displacement and /or substitution of essential metal ions from biomolecules and functional cellular units, (iii) the induction of conformational changes of polymers, and (iv) the influence on membrane integrity and transport processes [300]. In addition, highly toxic metals in a substrate could inhibit microbial growth, and thus decreases the bioleaching rate and efficiency [301]. A high content of carbonate in the solid residue, increases the pH of the leaching solution and influences microbial growth on substrates such as fly ash [290].

Particle Size, decreasing particle size leads to an increase in surface area resulting in an increase in the contact area between the leaching agents and the solid particles; as a result there is an increase in the leaching yield. Research has revealed that the highest solubilisation rate occurs with a particle size of a few tens of microns [271]. The solid liquid ratio's used is another factor. Increasing the solid mass causes an increase in the amount of a toxic metals in the leaching environment thus, an optimum pulp density must be determined for the bioleaching process [271, 296]

Bioleaching Period, bioleaching requires a longer period to leach metals when compared with chemical leaching i.e., *Thiobacilli* is a slow growing bacterium that may require a few weeks to complete the bioleaching process. Fungi generally show a shorter lag phase and hence may bioleach at a faster rate [293].

Physicochemical factors,other physical factors include shaking, and aeration. Most of these remaining factors are interconnected [186].

2.5.4 Fertiliser or compost production from MSW

MSW is composed largely of kitchen and yard waste; these wastes have been composted by many municipalities [302]. The composting process converts organic waste material into a low cost product, that is suitable for agriculture [303]. For compost production, many factors have to be taken into the consideration such as economic and environment, municipal landfill capacity; costs associated with land filling and transportation of materials; adoption of legislation to protect the environment; decreasing the use of commercial fertilisers; increasing the capacity for household waste recycling and improved quality of compost products [304].

The main advantages of compost production are: reduction in volume of the wastes, kill pathogens, prevents germination of weeds in agricultural fields, and destroys malodorous compounds [305]. There is rising interest in organic composter production from MSW for agricultural use due to positive effects on biological, physical, and chemical soil properties(Iglesias-Jiménez and Alvarez, 1993; Hargreaves*et al*., 2008).

Physical soil properties,the primary benefits of MSW compost is that it has a high content of organic matter and low bulk density [306]. MSW compost contains a humic acid to fulvic acid ratio of 3.55 [307]. MSW compost has some other advantages such as increasing soil organic matter; increasing soil C/N ratio; [308]; the compost has a higher water holding capacity than the soil; it improves soil structure [306]; and increases aggregate stability [309].

Biological soil properties soil quality is determined by soil microbiological properties [310]. Addition of MSW compost to the soil increases N, C and S immediately and for up to one month, while for P, biomass requires five months [311]. Other advantages of adding compost are an increase in soil microbial biomass and soil respiration (an index of general metabolic activity of soil microorganisms) [312]. Another measure of soil microbial health is the activity of soil enzymes involved in the transformation of the principal nutrients [313]. Research has shown that trace metals have an effect on the biological activity in a soil after being applied with compost derived from MSW, due to the high level of trace metals, this effects depends on the time of application, their concentration, and soil characteristics [313]. To study the effects of MSW compost on soil biology should include metal analysis [310].

Chemical properties

pHApplying MSW helps to increase soil pH and has been highlighted as a major advantage. This increase in soil pH is due to the mineralization of carbon and the subsequent production of OH ions by ligand exchange as well as the introduction of basic cations, such as K^+, Ca^{2+}, and Mg^{2+}[314].

Electrical conductivity EC and salt effects, increasing salt content has a negative effect on soil which effects plant growth, the EC of the soil solution relates to the dissolved solutes content and salt content in the soil. Agricultural soils EC levels range from 0 to 4 dS/m, while MSW composts range from 3.69 to 7.49 dS/m [315]. Applying MSW compost at rates ranging from 40 to 120 Mg/ha has led to an increase in the EC content of soil EC [308].

Nutrients (N,P, and K),differences in leaching rate or availability for plants depends on the feedstock and

compost maturity (Ring and Warman, 2000). MSW compost contains nitrogen which could become available for the plant, the availability of N in MSW compost has been estimated at 10% in the first year after application with some reports of N release in the second year after application(Zhang*et al.*, 2006; Hargreaves*et al.*, 2008). Some other study reported N in MSW compostcould be available 6 months after application [316]. While, for P from MSW compost requires three consecutive years [317]. Studies have reported 10-50% P in MSW being available during both the first and second years after application [306].

Phosphorus and nitrogen content in MSW compost are not regulated by the Canadian Council of Ministers of the Environment (CCME) or the United States EPA (USEPA, 2000; CCME, 2005)

Potassium is another important mineral for plant growth and was found to be increased even when low amounts of MSW derived compost was applied around 36–48% of total K in the MSW compost was found to be available to the plant [306]. The total concentration macronutrient and metals that has been found in MSW composts is shown in Fig 2.7.

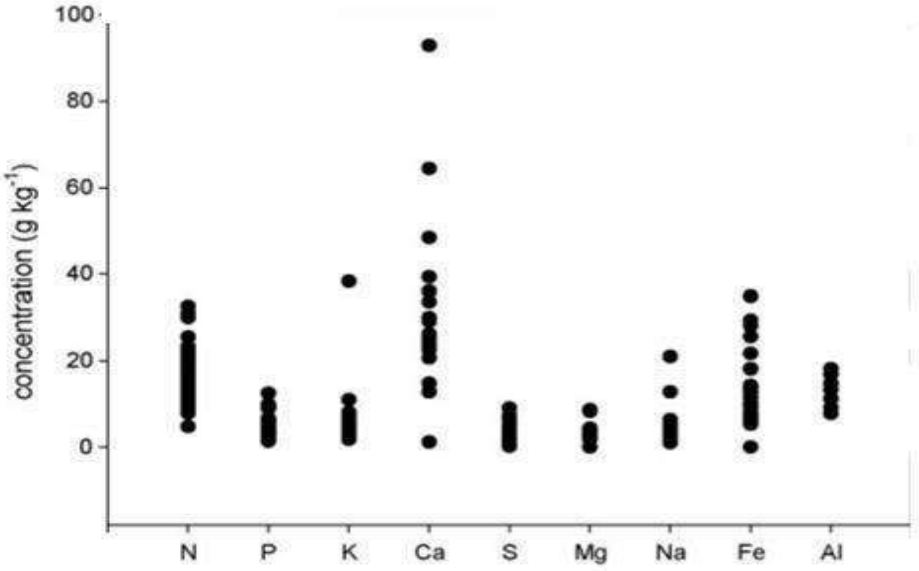

*Fig.2.7 Total concentrations of the macronutrients and metals present in MSW compost***[318]**

III. CONCLUSION

From the literature is clear that wastes especially municipal solid wastes can be used as a sustainable resource for bioenergy products such as biogas, biofuel, bioenzyme and biofertaliser. Generally, pre-treatment methods showed a significant increasing at bioenergy products,previous paper shown viability of MSW in bioenergy production published by[319-321].

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Heavy Metals in Some Lipstick products marketed in Makurdi Metropolis, Benue State Nigeria

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Abstract*— *Cosmetics still retain their attractive use and brilliant effects, however public concern about their toxicity has become a topic of debate. Trace amounts of toxic heavy metals can be either intentionally added to cosmetics or present as impurities in the raw materials. The present study reports the content of five heavy metals (Cd, Cu, Ni, Pb, and Zn) in six brands of lipstick products sold at various markets in Makurdi metropolis of Benue State, Nigeria using atomic absorption spectroscopy (AAS). Pb in the samples ranged from<0.01 – 3.92. mg/g, Zn ranged from 2.23 – 3.01 mg/g, Ni ranged from 0.12 – 0.23 mg/g and Cd ranged from <0.01 – 1.35 mg/g. all metals except Cd and Pb were above the safe limits for metals in products. Prolonged use of these products containing these metals therefore, may pose a threat to human health and could damage the environment. The results lead to the conclusion that constant control of metallic content in lipsticks and other facial cosmetics should be seriously considered.

***Keywords*— Lipstick products, Heavy Metals, AAS.**

I. INTRODUCTION

Cosmetics are substances intended to be used in contact with various external parts of the human body by means of rubbing, sprinkling or spraying on the desired part for the purpose of cleansing, beautification, altering appearance, correcting body odours and keeping the body in good condition (Lee et al., 2008). One of the most used cosmetics are lipsticks. Lipsticks are cosmetics containing oil, pigments, waxes and emollients that apply colour, texture and protection to the lips. They often come in varieties of colours. Lipsticks have water or gel base and may contain alcohol to help the product remain sticky. They temporarily saturate the lips with a dye and are usually designed to be water proof with many colours, shades and types of lipsticks available. As with most other types of makeup, lipsticks are typically but not exclusively used by women. Their use dates back to medieval time (Kumar *et al*., 2012).

Many lipsticks have been reported to contain heavy metals such as lead, arsenic, cobalt (Adepoju-Bello *et al.*, 2012) either as ingredients or impurities. Although the chemical constituents of cosmetics can sometimes be seen to raise eyebrows, some chemicals are widely seen and beneficial. Titanium dioxide TiO_2 found in sun screens and zinc oxides have inflammatory properties (Barakat, 2011). Mineral make-ups with these ingredients can have a calming effect on the skin which is particularly important for those with inflammatory problems such as rosacea. Zinc oxide is also anti-microbial, so mineral make-ups containing it can be beneficial for people with acne. However, the presence of other chemicals can spell danger to the consumers. Studies on animals show that if consumed in high amounts, it affects the kidney, stomach and liver. Mercury on the other hand is a neurotoxin and prolonged use of its products leads to inflammation of the liver, kidney and urinary tract. Lead can be very harmful even at low concentrations causing learning disabilities, behavioural issues, decreased muscle growth, brain damage among others(Ramakant *et al*., 2014).

In general, the use of some heavy metals in cosmetics has been controversial due to the biological accumulation of those metals and their toxicity in the human body (Patel, 2016).At low concentrations, heavy metals have been reported to have negative effects on consumers with acceptable limits of heavy metals varying according to the sub-population of interest (Ramakant *et al*., 2014). Most of

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these metals are not intentionally used as ingredients during the production of these cosmetics. They are simply impurities in the products and are not required to be listed on the labels. Manufacturers are however supposed to take care of these impurities but more often than not, these guidelines are so laid-back very few manufacturers remove these heavy metals from their final products. The metals of primary toxicology concern in cosmetics products include lead, mercury, cadmium and antimony. Dermal exposure is expected to be the most significant route for cosmetics since majority of cosmetics are applied via the skin. Oral exposure can also occur for cosmetics used in and around the mouth (Ramakant *et al*., 2014).

Lead, cadmium, mercury, chromium, nickel, and copper are the most common heavy metals detected in cosmetic products, including shampoo, lipstick, cream, eye shadow and powder (Volpe *et al*., 2012). The ingredients and colorants, along with inadequate purification of raw materials, contribute to the presence of these impurities in cosmetics (Al-Saleh *et al*., 2009). Cosmetics appear on the list of products manufactured in various parts of the world for which recall notices have been issued in the US. Thus, in Caribbean countries, an import alert was declared for skin-whitening cream after Hg level in the product measure 8% (Grosser *et al*., 2011).

In recent times, cosmetic application has become a way of life as many women and even men want beauty enhancement aids. However, only a handful are concerned if they contain toxic chemicals and potentially harmful ingredients (Adepoju-Bello *et al.*, 2012). Even with the regulation of many cosmetic products, there are still health concerns regarding the presence of such chemicals in them. Since cosmetic use is rapidly increasing worldwide and various heavy metals are usually found in then, health hazards are posed to their consumers as they are vulnerable due to lack of awareness. This work was therefore aimed to evaluate the content of some heavy metals which include lead, zinc, copper, cadmium and nickel present in some lipsticks sold in local markets within Makurdi, Benue State, Nigeria.

II. STUDY AREA

This study was carried out in Makurdi Local Government area of Benue State, situated at 7.74^0 North Latitude, 8.51^0 East longitude and 104 meters elevation above sea level with a population of about 292,645 inhabitants.

Sample collection

Samples of lipsticks were randomly sampled from retailers from four markets including High level, Wurukum, Wadata and Northbank markets.At each market,three samples were obtained and coded before been taken to the laboratory for further analysis.

SAMPLE CODE	LIPSTICK BRAND	COLOUR	LOCAL MARKET
A	Romantic Shiny	Purple	Wurukum
B	Beauty Lady Rose	Light Brown	Wurukum
C	Black Opal	Pink	Wadata
D	Lovely Absolute	Red	Northbank
E	Miss Rose	Pitch	High Level
F	Cisou	Oxblood	Wadata

Sample Digestion and Analysis

Sample digestion for the determination of lead, zinc, copper, cadmium and Nickel was done as described by Sonawane *et al.* (2013).]. Exactly 1.0g of each sample was weighed into a conical flask and 10 mL mixture of concentrated acid HNO_3: $HCLO_4$ (3:1) added. This was then heated for 3 hours on a hot plate at 90^0C before 5.0 mL of acid mixture was added and the solution heated again for 2 hours to complete the digestion. The samples were cooled and made up to 25.0 mL in volumetric flask. The solution was finally filtered through Whatman no. 41 filter paper, and the clear solution was used for metal quantification using AAS (model: Buck Model 210 VGP)

III. RESULTS AND DISCUSSION

The concentrations of various metals in the sampled lipsticks from Makurdi metropolis in Benue State is given in Figures 1,2,3,4 and 5.

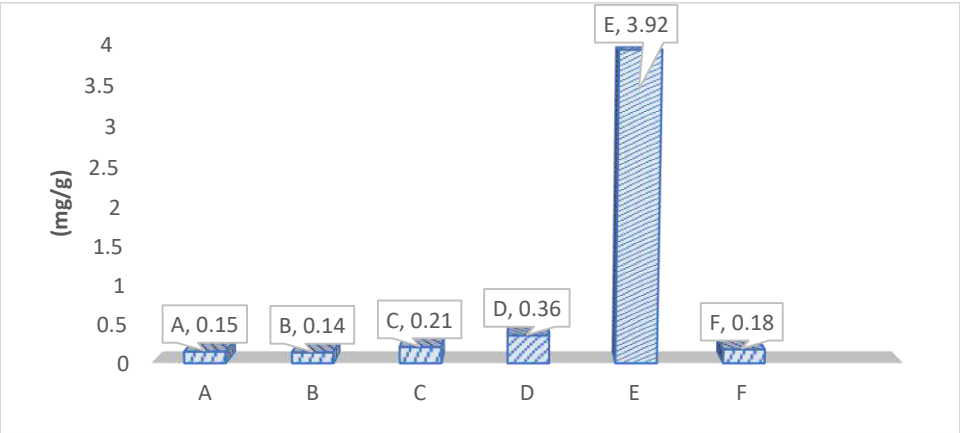

Fig.1: Cu concentration in various lipstick samples

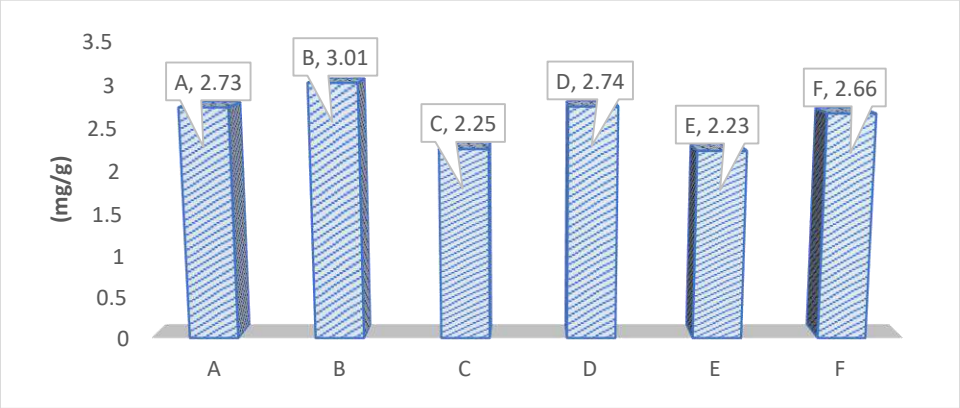

Fig.2: Zn concentration in various lipstick samples

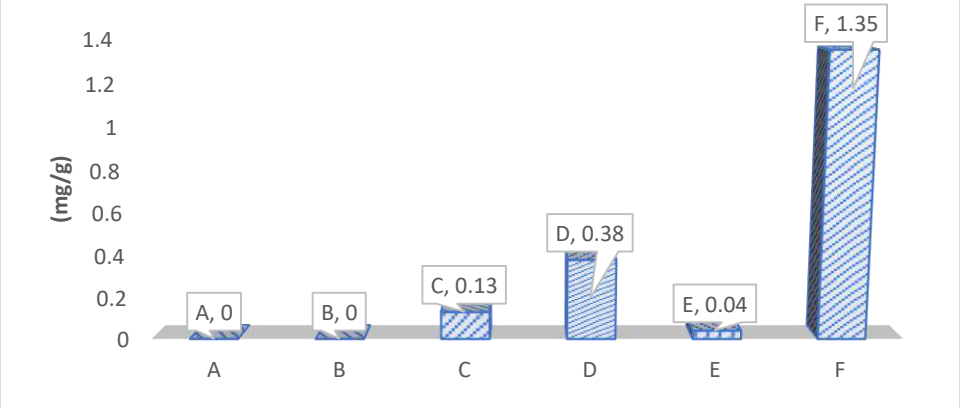

Fig.3: Cd concentration in various lipstick samples

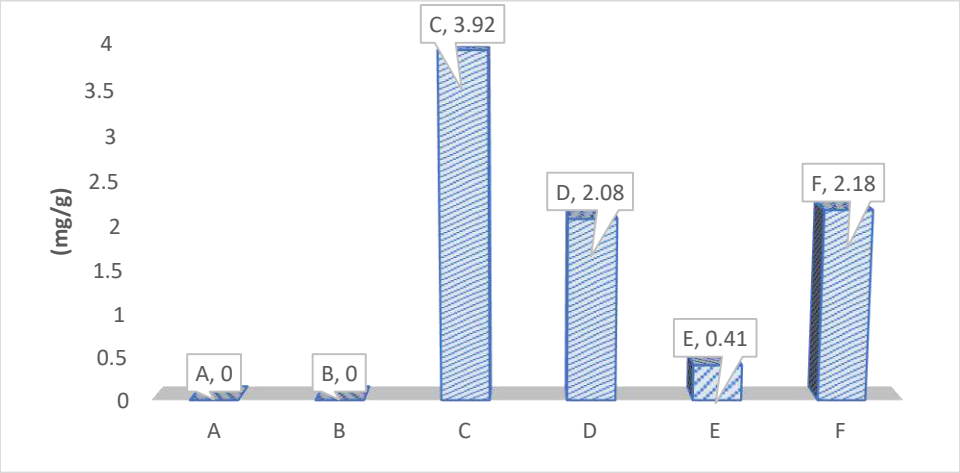

Fig.4: Pb concentration in various lipstick samples

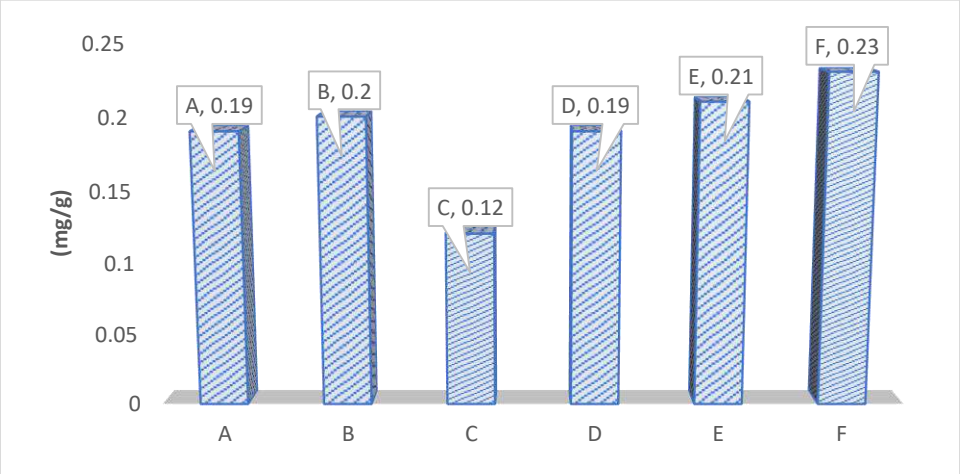

Fig.5: Ni concentration in various lipstick samples

The concentration of Pb in the samples ranged between <0.01 – 3.92. mg/g. Two of the samples were not detected with lead. This conforms with the European union act which prohibits the preserve of lead in lipsticks/cosmetics. The values of the samples that were detected with Pb were less than permissible value 20 mg/g.The concentration of Zinc ranged between 2.23 – 3.01 mg/g with a standard deviation of 0.22684 and copper 0.13 – 3.92mg/g and its values were less than 50 mg/g which is the standard for Cu in cosmetics. Zinc is efficient in smaller quantities but even at that, bioaccumulation can pose potential heaths damages.Ni concentration in the six samples ranged between 0.12 – 0.23 mg/g. The values of the detected samples were less than 5 mg/g which is the standard limits.Cadmium in some samples was not detected. Its concentration ranged between <0.01 – 1.35 mg/g. The European Union and Health Canada also prohibits the presence of cadmium in cosmetics. The samples detected with Cd were less than 15 mg/g.

This study showed that the concentration of Cu which ranged between 0.14 – 3.92 fell under the permissible limits. Zinc ranged between 2.23 – 3.01 mg/g was within, Cd and Pb fell below the permissible limit and ranged between 0.00 – 1.35 mg/g and 0.00 – 3.92 mg/g respectively; while Ni ranged between 0.12 – 0.23 mg/g was within permissible limits. The concentrations of these heavy metals for all the samples fell within their permissible limits.The maximumvalue for Ni (0.23 mg/g) was found to be higher than that reported inmost previous studies (Al-Saleh et al., 2009; Barakat, 2011). The highest concentration of Pb (3.92 mg/g) wasfound to be higher than the reported values (Al-Saleh*et al*., 2009; Saeed *et al*., 2010; Barakat, 2011). The maximum valueof Zn (3.01

mg/g), which as an oxide has properties similar to TiO_2, was found tobe lower than many reports in the literature.

The results of the analysis showed the presence of copper, zinc nickel in 90% of the samples. The concentration of heavy metals in the lipstick samples indicate that the control measures for these elements must be maintained in other for the lipsticks to be safe for use. The differences in concentration of the various metals would be due to the variations in materials used to get each colour of lipstick. Also, the lipsticks may be graded into high class and low class, which determines the quality of the materials used for each production. Zainy (2017) reported that higher class oflipsticks (more expensive, higher quality) is safer than the lower class (less expensive,lower quality), and is consistent with earlier research for lead and cadmiumlevels in various cosmetic brands.

These heavy metals in lipsticksare impurities and bind with proteins incells, leading to cell death and multiple diseases (Shanker, 2008). The slow liberation of these metals into thebody means that they may cause damage after accumulating over time in variousorgans. Other studies have also reported heavy metal concentrations invarious cosmetic products (Adepoju-Bello *et al.*, 2012).

IV. CONCLUSION

The application of atomic absorption spectroscopy (AAS) technique allowed the quantification of heavy metals in lipsticks. In some samples, lead and cadmium were present at level prohibited by European regulation. It should be emphasized that although Pb and Cd were not detected in some samples due to the detection limit of the analytical procedure, this does not mean that these metals were completely absent.

However, from the results one can deduce that a lower amount of Pb, Ni and Cdis more beneficial for consumers, especially for those with hypersensitivity to contact allergies. Consequently, extensive uses of such products should be avoided. Thus, there is need for further assessment of risk to human health from exposure to cosmetics that are contaminated with heavy metals. Careful selection of the raw materials used in producing them with regard to heavy metal content can improve the safety of cosmetics and their impact on the environment.

Conflict of interest: There is no conflict of interest

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Utilization of entrepreneurial information among rural women farmers in Akinyele Local Government Area Oyo State

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Abstracts— *Entrepreneurship on a small scale is the only solution to the problems of unemployment and proper utilization of both human and non-human resources and improving the leaving conditions of the poor masses. Therefore, Utilization of entrepreneurial information among rural women farmers in Akinyele Local Government Area Oyo State was investigated. 200 respondents with the aid of well structured questionnaire were selected through Multi-stage sampling technique. Data were analyzed using frequency counts, percentage and means and PPMC at 0.05% level of significance. The result of analysis revealed that most 45.5% of respondents are in their active age, married with majority (41.5%) had farming experience between 16 years and above. Also, utilization of entrepreneurial information among women farmers is high. However, Securing working capital, lack of transportation, lack of information and delay of payment, high cost of labor and high cost of inputs are major constraints faced by rural women farmers in the study area. PPMC Analysis reveals that there is significant relationship between constraints faced by rural women farmers and utilization of entrepreneurial information (r-value =0.365and p- valve = 0.000).It is therefore recommended that Rural women farmers should be introduced to the internet, in order to get more information on entrepreneurship. And also, various tiers of government should create programs that will catalyze entrepreneurial development with the aid entrepreneur information (with special focus for women) in the rural areas.*

Keywords— ***Utilization, Rural, Women farmers, Entrepreneurial information.***

I. INTRODUCTION

Rural women are active agents of economic and social change and environmental protection who are, in many ways and to various degrees, constrained in their roles as farmers, producers, investors, caregivers and consumers. They play crucial roles ensuring food and nutrition security, eradicating rural poverty and improving the well-being of their families yet continue to face serious challenges as a result of gender based stereotypes and discrimination that deny them equitable access to opportunities, resources, assets and services. Women are the backbone of the rural economy, especially in the developing world. Yet they receive only a fraction of the land, credit, inputs (such as improved seeds and fertilizers), agricultural training and information compare to men. Empowering and investing in rural women as been shown to significantly increase productivity, reduce hunger, and malnutrition and improve rural livelihood.

Information utilization is particularly important to entrepreneur's final decision because information is deemed to be worthless if it is not put to good use (Ottun and Moore 1997), Suggest that information utilization be conceptualized in terms of type and extent of usage in the decision making process. Information is the powerful knowledge resource that can enhance competitive advantage. In particularly information pertaining to customers and competitors are crucial towards the development of market orientation. Information acquisition and utilization is an important activity particularly salient for firms that have high levels of

entrepreneurial orientation. Information on entrepreneurship should not just be gathered, but should be well used. Utilization and acquisition of entrepreneurial information is very important for an excellent performance in business.

(According to Pleter (2005), the entrepreneurs are business people who build (start), develop and manage a business, risking time, efforts and money to this purpose. The entrepreneurship as a concept refers to an ability of the individual to put into practice an idea possessing some qualities such as creativity, innovation, risk taking, and ability to plan and manage the activities in view of fulfilling the proposed goals. This term knows different approaches at the level of each state, being influenced by certain elements such as education, culture, and environment, legislative and political system (Piti, 2010). The managerial and self-control qualities of the entrepreneur have evolved as two new major dimensions of their personality, in addition to the traditional concept that the entrepreneurs are involved in risk taking, that is, they are innovative and creative (Biswas, 2000). The recognition and evaluation of business opportunities represents the beginning of the entrepreneurial process (Baron and Henry, 2010). The individual entrepreneur detects or creates business opportunities that he then exploits by small and medium size enterprises, usually taking part in the financing of capital for that company, or merely, "he sells" the idea of the business project (Cuervo et al, 2010). It is against this backdrop that this research investigated the extent of Utilization of entrepreneurial information among rural women farmers in Akinyele Local Government Area Oyo State with the following specific objectives;

1. To identify the socio economic characteristics of the respondent in the study area.
2. To assess the utilization of entrepreneurial information among rural women farmers in the study area.
3. To ascertain the constraint faced by rural women farmers in the utilization of entrepreneurial information in the study area.

Hypothesis of the study

Ho_1 – There is no significant relationship between constraints faced by rural women farmers and utilization of entrepreneurial information in the study area.

II. MATERIALS AND METHODS

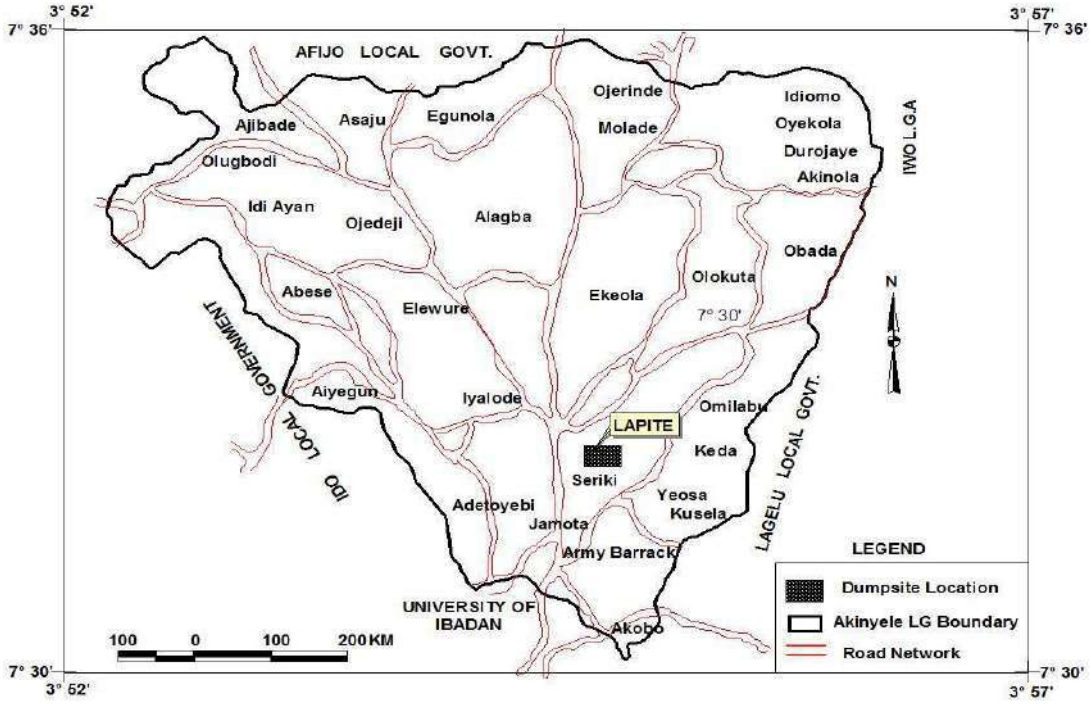
The study was carried out in Akinyele Local government area which was created in 1976 with the administrative headquarters located at Moniya. The local government shares the same boundaries with Afijo local government to the north, Lagelu local government area to the east, Ido local government area to the west and Ibadan north local government area to the south. It occupies a land area of 464.892square kilometers with a population density of 516 persons per square kilometer. Using 3.2% growth rate from 2006 census figures, the 2010 estimated population for the local government is 239,745. It is dominated by the Yoruba's among other resident tribes such as Ibo, Tiv, Hausa, Nupe, Fulani etc. The residents are of Christianity, Islamic, and traditional religion. The L.G.A. is endowed with fertile agricultural land suitable for the cultivation of crops like orange, mango, banana, pineapple, cassava, yam etc. The area is also notable for palm oil production.

Sampling Procedure and Sample size

Multistage sampling techniques were used to select respondents in the study area. First stage involved randomly selecting six (6) out of twelve wards in Akinyele local government area, Oyo state, Nigeria. Second stage involved purposively selecting twenty (20) villages/communities from the randomly selected wards. Third stage involved randomly selecting Ten (10) respondents in each of the twenty (20) purposively selected villages/ communities, which account for a total of two hundred (200) respondents used as sample size for the study.

Data Analysis

Descriptive statistics such as frequency distribution, percentage was used to analyses all objectives while PPMC for hypothesis of the study.



III. RESULTS AND DISCUSSION

Socio-economic characteristics of respondents

The result of analysis in Table 1 shows that 45.5% of the respondents fall within the age range of 31-40 years age bracket, 25.0% were between the age range of 41-50, 22.5% follows between the age range of 50 and above, while only 7.0% between the age range of 20-30. The result shows that the range of 31-40 have the highest percentage; this implies that most of women farmers are still in their active age. This agrees with the finding of *Odebode (2008)* who reported that perception and acceptability of innovation is mainly associated with youthful and active age of farmers. Also, the table shows that (85.5%) of the respondents were married, (7.5%) were widowed, (6.5%) were single, while (0.5%) were divorced. This is supported with the findings of *Adelore et al (2006)* that most farmers are married. Based on their educational level, result shows that (28.0%) of the respondents has primary education (23.5%) of the respondents has adult education, (20.0%) of the respondents had no formal education, (19.5%) of the respondents had secondary education, and (9.0%) of the respondents had tertiary education. This implies that majority of the women farmers within the study area are not well educated. Furthermore, Table 1 (55.5%) of the respondents were Christians while (44.5%) of the respondents were Muslims. More so, the result also shows that respondents with household size of 1-4 were (38.0%), 5-8 were (52.5%) while 9 and above were (9.5%). This implies that the larger the household size the more labor availability and the more income requirement to meet household needs. Also, (73.0%) 0f the respondents had their secondary occupation as trading. This means they have another job apart from being a farmer which can be used to generate more income into the family. The result above further shows that, (4.0%) of the respondents had farming experience between 1-5years, (23.5%) had farming experience between 6-10years, (31.0%) had farming experience of between 11-15years, while (41.5%) had farming experience between 16 years and above.

Table 1: Socio economic characteristics of the respondents

Variable	Frequency	Percentage
Age		
20-30	14	7.0
31-40	91	45.5

41-50	50	25.0
Above 50	45	22.5
Marital Status		
Single	13	6.5
Married	171	85.5
Divorce	1	0.5
Widow	15	7.5
Educational Level		
Adult education	47	23.5
No formal education	40	20.0
Primary education	53	28.0
Secondary education	39	19.0
Tertiary education	18	9.0
Religion		
Christian	111	55.5
Islam	86	44.5
Traditional	3	1.5
Household size		
1-4	76	38.0
5-8	105	52.5
9 above	19	9.5
Secondary occupation		
Farming	21	10.5
Trading	146	73.0
Teaching	15	7.5
Others	18	9.0
Farming experience		
1-5	8	4.0
6-10	47	23.5
11-15	62	31.0
16 above	83	41.5
Total	**200**	**100**

Source: field survey 2018

The result shows that majority of the respondents 71.5% and 59.5% respectively rely heavily on the information acquired on entrepreneurship and make use of different types of support that is offered to people who want to start their own business respectively. This implies that knowledge is shared out so as to acquire more because no man is an island of knowledge. Also, the table shows that 29.5% of the respondents never delegates tasks and responsibilities to their employees.Also, 85.5% of the respondents persist in the face of their adversity because an entrepreneur is confronted with

various risks and an entrepreneur that cannot persist in the face of adversity may likely not succeed. So also, 48.5% and 46.0% of the respondents manage the financial records of their business and maintain the record respectively. This implies that it will be easier to identify if they are running at lose or making profit.Also, 69.0% of the respondents were able to determine the competitive price of their products. More so, 83.5% of the respondents had effective advertising skills for their products. Gorman et al (2004) observed that for every entrepreneur to succeed in business, such entrepreneur must have effective marketing skills. Again, 86.5% of the respondents had effective marketing skills. Marketing is the process of getting consumers interested in your company's product or service. This goes in line with Mazur (2005) marketing is the delivery of a standard of living to the society. Furthermore, 31.0% of the respondents exchange entrepreneurial information with others. 35.0% and 32.0% of the respondents respectively develop and maintain favorable relationship with successful entrepreneurs. Also, 89.0% of the respondents have excellent communication ability. Nwagwugwu and Okoye (2009) good communication planning promotes every business ventures thereby, making such a business to be successful. This also goes in line with what Denyer (2011) perceived that communication ability is dependent on one's ability and skill to listen, read, write and speak.82.5% of the respondents are committed to their work. Also, 90.0% of the respondents are of great confidence. 65.0% of the respondents are risk taker. This goes in line with David (2008) that every business entails great risk. 69.0% of the respondents have a high energy level for entrepreneurship. Furthermore, 85.5% of the respondents are willing to learn about entrepreneurship development. These in line with the findings of Amesi (2009), if we can control and coordinate ourselves effectively in attending to entrepreneurs programs, then we are sure of achieving success. Finally 73.0% of the respondents embrace new innovations.

The table 2b above shows that utilization of entrepreneurial information among women farmers is high with 55.0%. This means that majority of the women farmers in the study area are utilizing the information they get from entrepreneurship development to boost the economy.

Table 2a: Utilization of Entrepreneurial Information among Rural Women Farmers

UTILIZATION OF INFORMATION	**ALWAYS**	**OFTEN**	**SOMETIMES**	**RARELY**	**NEVER**
I rely heavily on the information acquired on entrepreneurship.	143(71.5)	27(13.5)	19(9.5)	7(3.5)	4(2.0)
I make use of the different types of support that is offered to people who want to start their own businesses.	119(59.5)	48(24.0)	32(16.0)	1(0.5)	0(0.0)
I delegates tasks and responsibilities to employees in my business.	48(24.0)	45(22.5)	3(1.5)	45(22.5)	59(29.5)
I persist in the face of my adversity.	171(85.5)	18(9.0)	10(5.0)	1(0.5)	0(0.0)
I am able to maintain the financial record of my business.	97(48.5)	92(46.0)	5(2.5)	4(2.0)	2(1.0)
I am able to manage the financial assets of my business.	97(48.5)	90(45.0)	13(6.5)	0(0.0)	0(0.0)
I am able to determine the competitive price of my products.	138(69.0)	51(25.5)	11(5.5)	0(0.0)	0(0.0)
I have effective advertising skills.	167(83.5)	21(10.5)	9(4.5)	2(1.0)	1(0.5)
I have effective marketing skills.	173(86.5)	21(10.5)	3(1.5)	2(1.0)	1(0.5)
I exchange entrepreneurial information with others.	49(24.5)	48(24.0)	62(31.0)	37(18.5)	4(2.0)

I develop and maintain favorable relationship with successful entrepreneurs.	70(35.0)	64(32.0)	43(21.5)	21(10.5)	2(1.0)
I have excellent communication ability.	178(89.0)	18(9.0)	4(2.0)	0(0.0)	0(0.0)
I am very committed to my work.	165(82.5)	30(15.0)	4(2.0)	1(0.5)	0(0.0)
I am very self-confident.	180(90.0)	12(6.0)	5(2.5)	2(1.0)	1(0.5)
I am a risk taker.	130(65.0)	66(33.0)	3(1.5)	1(0.5)	0(0.0)
I have a high energy level.	138(69.0)	49(24.5)	13(6.5)	0(0.0)	0(0.0)
I am willing to learn about entrepreneurial information continually.	171(85.5)	21(10.5)	5(2.5)	3(1.5)	0(0.0)
I embrace new innovations.	146(73.0)	35(17.5)	18(9.0)	1(0.5)	0(0.0)

Source: Field Survey, 2018.

Table 2b: Categorization of Respondents based on Utilization of Entrepreneurial Information among Rural Woman farmers.

Categorization	**Frequency**	**Percentage**
High	110	55.0
(Above mean)		
Low	90	45.0
(Below mean)		
Total	**200**	**100**

Mean =27.0

Source; Computed from Researcher's Survey, 2018.

The result in table 3 shows that 87.0% and 86.5% of the respondents respectively stated that securing working capital and insufficient financial assistance by financial institution has been a major constraint in utilizing entrepreneurial information in the study area. This implies that there is no adequate funding either by government or cooperative societies and it makes farmers unable to utilize entrepreneurial information. Also, 48.0% of the respondents stated that lack of transportation facilities is a major constraint they face. This implies that there is bad transportation network in our rural areas and this always leads to spoilage of products in the process of transporting it to the urban areas while 41.0% of the respondents also lack market information. Furthermore, the high cost of labor 78.0% has been a major constraint in utilizing entrepreneurial information. This implies that there is limited number of labors or sometimes limited skilled labor to perform the practice effectively. Also, 71.0% of the respondents in the study area are faced with gender inequality problems. This implies that, in many cases the women entrepreneurs also faced non acceptance from domestic front. Their family members especially the males of the family were not ready to digest the fact that the women were earning more than them and stepping out of the house. Gender inequality exists in terms of economic development as well as the rates of entrepreneurial activity. This goes in line with the findings of Kelley (2011) A GEM study of 18 economies from 2002 to 2010 suggests that women's entrepreneurial activity is lower than that of their male counterparts at different stages of development. 83.5% of the respondents in the study area deduced that unfavorable policies made by the government are major constraints. Lastly, 74.0% of the respondent had low education. This implies that the illiteracy of the farmers has hindered them from effectively utilizing entrepreneurial information.

The table 3b shows that majority 55.0% of the respondents are faced with high constraints while 45.0% of the respondents with low constraints.

Table 3a: Constraints Faced by Rural Women Farmers in Utilizing Entrepreneurial Information.

Constraints	Major constraints	Minor constraints	Not a constraints
FINANCIAL			
Securing working capital.	174(87.0)	24(12.0)	2(1.0)
Insufficient financial assistance by financial institutions.	173(86.5)	19(9.5)	8(4.0)
Inadequate loan.	167(83.5)	31(15.5)	2(1.0)
Entire loan is not given at a time.	167(83.5)	25(12.5)	8(4.0)
Low price for the produce.	90(45.0)	105(53.0)	4(2.0)
Lack of means of production.	119(59.5)	78(39.0)	3(1.5)
MARKETING			
Lack of information and network.	82(41.0)	74(37.0)	44(22.0)
Lack of transportation facilities.	96(48.0)	50(25.0)	54(27.0)
Lack of market information.	82(41.0)	44(22.0)	74(37.0)
Delay of payments.	95(47.5)	69(34.5)	36(18.0)
PRODUCTION AND LABOR			
High labor cost	156(78.0)	40(20.0)	4(2.0)
Non availability of skilled workers.	138(69.0)	61(30.5)	1(0.5)
High cost of inputs.	155(77.5)	39(19.5)	6(3.0)
PERSONAL/ GENERAL			
Gender inequalities.	142(71.0)	33(16.5)	25(12.5)
Unfavorably policies.	167(83.5)	32(16.0)	1(0.5)
Health problem	143(71.5)	50(25.0)	7(3.5)
Unfriendly institution that governs the everyday business and generally in accessibility of policies.	160(80.0)	33(16.5)	7(3.5)
Low education.	148(74.0)	38(19.0)	14(7.0)

Source: Computed From Researcher's Survey, 2018.

Table 3b: Categorization of Respondents Based on Constraints Faced by Rural Women Farmers in Utilizing Entrepreneurial Information

Categorization	Frequency	Percentage
High (Above mean)	110	55.0
Low (Below mean)	90	45.0
Total	**200**	**100**

Mean= 26.5

Source: Computed From Researcher's Survey, 2018.

Table 4.10: Ho3: Relationship between constraints faced by rural women farmers and utilization of entrepreneurial information.

VARIABLES	r-VALUE	p-VALUE	DECISION
Constraints faced by rural women farmers and utilization of entrepreneurial information.	0.365	0.000	S

Source: Computed From field Survey, 2018.

The table 4 above shows that there is significant relationship between constraints faced by rural women farmers and utilization of entrepreneurial information (R-value =0.365and p- valve = 0.000). This is in line with the report findings of (Aculai et al., 2006; Aidis, 2006) when necessary resources are available to women entrepreneurs, women still hesitate to set up units or do not succeed in their ventures due to constraint imposed on them by their immediate environment such as family commitments and lack of market opportunity.

IV. CONCLUSION AND RECOMMENDATIONS

Based on the findings of the study, the following conclusions were drawn: The study reveals that majority of the respondents' belonged to young age group and have highest percentage of married women. It also explains that most of the respondents utilized every information they got on entrepreneurial development and are willing to learn about entrepreneurship information continually. However, Securing working capital, lack of transportation, lack of information and delay of payment, high cost of labor and high cost of inputs are major constraints faced by rural women farmers in the study area. Conclusively, it can be deduced from the study that most of the women farmers are familiar with what entrepreneurship is, but they are hindered, hampered, and incapacitated due to the above mentioned constraints. It is therefore recommended that Rural women farmers should be introduced to the internet, in order to get more information on entrepreneurship. And also, Various tiers of government should create programs that will catalyze entrepreneurial development with the aid entrepreneur information (with special focus for women) in the rural areas.

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Evaluating the effect of adding vitamins E & C to the extender for Barki ram semen by cooling

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Abstract— *The present work aimed at evaluating adequacy of adding different non-enzymatic additives; i.e. vitamins E and C, in to semen diluent on physical and morphological characteristics as well as oxidative status of ram semen stored at 4°C. Ejaculates (n=80) were collected from Five sexually mature Barki rams during the period from January to April. After initial evaluation, each adequate raw ejaculate was diluted (1:10) with Tris-citrate egg yolk extender. Each diluted ejaculate was further split into seven aliquots to evaluate addition of three levels of either ascorbic acid or alpha tocopherol (0.1, 0.2 or 0.3 mM for each treatment) against control (untreated) specimens for their ability to maintain sperm viability criteria over 48 hr (T_0 and T_{48}) of chilled storage at 4 °C.The changes in seminal plasma oxidative status indices as well as enzymatic activates were also determined throughout the period of cooled storage.The results showed that addition of adequate concentrations of ascorbic acid (0.1 mM) or α-tocopherol (0.3 mM) to semen diluent can reduce ($P<0.05$) the oxidative stress and, hence,maintained($P<0.05$) physical characteristics of ram spermatozoa compared to controlthroughout the 48 h period of cold storage.*

Keywords— ***Ram semen; sperm diluent; ascorbic acid; alpha tocopherol.***

I. INTRODUCTION

Generally, liquid-chilled preservation of semen has been reported to reduce the fertilizing capacity of spermatozoa along with both sperm motility and morphology over time of storage (Perumal *et al*., 2013)as results of accumulated reactive oxygen species (ROS) which leads to the death of the sperm cells (Amidi *et al*., 2016). The development of assisted reproductive techniques, including artificial insemination (AI) and in vitro fertilization (IVF), require improving spermatozoa physical characteristics by supplementing the diluent with a variety of antioxidant substances such ascorbic acid (Vitamin C) and α-tocopherol (vitamin E), which were reported toplay important roles as non-enzymatic antioxidant for spermatozoa (Abd El-Hamid *et al*., 2018; Zeitoun and Al-Damegh, 2015),by protecting sperm cells from generated ROS that induce damage to sperm DNA over preservation time (Alvarez and Storey, 2005; Dandekar *et al*., 2002).Further, both ascorbic acid and α-tocopherol were used to reduce ROS related testicular impairments in animal tissues (Acharya *et al*., 2008), and improve the antioxidant enzymatic activity of seminal fluid (Foote *et al*., 2002), thereby enhancing sperm motility and viability during semen processing (Soren *et al*., 2016). Therefore, the present study aimed to investigate the effect of supplementing semen diluent with different levels of Ascorbic acid (Vitamin C) or α-tocopherol(Vitamin E) on maintaining ram sperm traits during 48 h of chilled preservation at 4 °C,as well asdetermination of some antioxidant enzyme activities throughout the period of chilled storage.

II. MATERIALS AND METHODS

This study was carried out at Artificial Insemination Lab, Mariout Research Station located 34 km west of Alexandria and belonging to the Desert Research Center (DRC), Egypt.

Animal and management.

Five sexually mature Barki rams, aged from 2 - 3 years with averaged body weight of 45.0 ±2.0 kg were used in this study from January toApril. The animals were hosed in closed pens throughout the experimental period. The rams were fed concentrate according to their body weight

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requirements (NRC, 2007). All rams were given Egyptian clover *(Trifoliumalexandrinum)* hay as a roughage ration *adlibitum*. Each animal received 1 kg /day of pelleted concentrate that contained 65% total digestible nutrients (TDN) and 14% crude protein. Fresh water was presented twice daily. Before executing the study, all rams were examined clinically and were found to be free of any disease or reproductive disorders.

Semen extender.

A Tris-citrate egg yolk extender was prepared for dilution of ram semen according to El-Bahrawy *et al*. (2004). The extender was composed of Tris buffer (0.25 Mol, 3.025 %), sodium citrate (1.67 %), and was further supplemented with egg-yolk (2.5 %) plus a mixture of Penicillin procaine (500 iu/ml) and Streptomycin (500 µg/ml) (El-Bahrawy et al. 2004). The extender was freshly prepared 24 hr prior to the collection sessions.

Semen collection.

A total number of 80 ejaculates were collected from the rams twice weekly using artificial vagina as previously described (El-Bahrawy et al., 2004). Mean values of raw ejaculates' physical traits included in the study were volume 0.96 ± 0.06ml, progressive motility ≥ 90%, pH 7.2 ± 0.1, mass motility 4.26 ± 0.16 and sperm concentration 2374.4 ±26.2 ($\times 10^6$ sperm/ml). Each raw ejaculate was diluted (1:10) with Tris-citrate egg yolk extender, and was further divided into two major groups. The first major group of diluted semen was then split into three aliquots, where each was supplementedwith one of three levels (0.1, 0.2 or 0.3 m M) alpha tocopherol (Vit E, Sigma-Aldrich, St. Louis MO, USA).Another similar set of aliquots was supplemented with with the same three levelsbut of ascorbic acid (vitamin, Sigma-Aldrich, St. Louis MO, USA). All six supplemented spicemens were evaluated, throughout the period of the study against control (non-supplemented) group.(table1). All diluted semen groupswere evaluated for physical characteristics and some biomarkers of enzyme activities during 48 hr (T_0 and T_{48}) at 4 ^oC of cooling period.

Table.1: Different levels of vitamins E and C supplementation within ram semen diluent.

Treatments	Experimental groups and levels of each treatment			
	Control	L_1	L_2	L_3
Vitamin E	-	0.1 mM	0.2 mM	0.3 mM
Vitamin C	-	0.1 mM	0.2 mM	0.3 mM

Semen evaluation.

Total sperm motility was estimated in all diluted samples byusing a phase-contrast microscope (Leica) at 40 X magnification, where an average of 5 random fields was obtained to the nearest 5%. Sperm vitality (live and dead sperm, %) were examined using the differential staining technique, where a mixture of 10 µl of semen and 5 µl of freshly-prepared eosin-nigrosin stain was smeared on a warm stage, and were examined under high power magnification (100×). Sperm abnormalities and acrosome integrity were evaluated using Romanowski's triple-stain technique (DIFF-QUICK III, Vertex, Egypt). Smears preparation and staining procedure were conducted following instructions provided by the manufacturer, and the stained smears were evaluated using a phase-contrast microscope at 100x magnification. Sperm plasma membrane integrity was determined by the hypo-osmotic swelling test (HOST) as described by Mosaferi *et al*. (2005), where at least 200 sperm were evaluated at 40 X magnification.

Seminal plasma biomarkers and enzymes activity assessment.

Seminal plasma samples were obtained during the cooling period (T_0 and T_{48}) and were subjected to biomarkers and enzymes activities using commercial kits. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) were analyzed using colorimetric kits (Spectrum, Egypt) according to Reitman and Frankel, (1975). Alkaline phosphatase (AKP), Lipid peroxide (LP) and hydrogen peroxidase (HP) were determined using colorimetric kits (Biodiagnostic, Egypt) according to Belfield and Goldberg, 1971; Koracevic*et al.* 2001 andAebi, 1984, respectively.

Statistical analysis.

of The changes in physical and morphometric sperm properties among different levels within each treatment (vitamins C and E), as well among control and the optimum concentration of each treatment during the cold storage period were analyzed by the General Linear Model (GLM) procedure (SAS, 2006) using the following model:

Yijk =μ + Li+Hourj+ L*Hourij + eijk

Yijk = observations, μ= Overall means, Li = effect of ith Levels (i: 1-4), Hourj = effect of jth Hour (j: 1-3), Hour*Lij = interaction between levels and hours.

eijk = Experimental error.

The statistical significance threshold was set at 0.05 and the differences between means were detected byDuncan's multiple range test..

III. RESULTS

Effect of different levels of Vitamin E on chilled semen traits

Means values ± SEM of physical characteristics sperm including sperm motility (%), normal sperm (%), sperm abnormalities (primary and secondary, %) acrosome integrity (%) and hypo-osmotic swelling test (HOST, %) are presented in table (2).

Overall mean of sperm motility (%) was significant higher (P<0.05) in level one (L_1) and level two(L_2) of vitamin E (87.66, 92.22 ± 0.93 %, respectively) compared with both level three (L_3) (87.00±0.93%) or control (85.66±0.93%)at 48 of chilled preservation at 4°C. There was a significant interaction (P<0.05) between time of preservation and levels were recorded. The higher (P<0.05) value of sperm motility was recorded in all levels of vit (E) at T_0 of chilled preservation, while the low value was recorded in control (66.00±1.00 %) at T_{48} of chilled preservation at 4°C (table 2).Mean value of normal sperm (%) was significantly higher (P<0.05) in (L_1) and (L_2) of vitamin E (93.77, 92.22 ± 0.57 %, respectively) followed byL_3(90.33±0.57%) compared with control (79.88±0.57%)at$T_{48}$of chilled preservation. Moreover, the higher (P<0.05) value of normal sperm was recorded in all levels of vit (E) at $T_0$of chilled preservation, while the low value was recorded in control (66.00±1.00 %) in T_{48} of chilled preservation at 4°C (table 2).

The results also showed that no significant was found among either levels of Vit. E and control groups in values of primary sperm abnormalities (%).However, the higher (P<0.05) values of primary sperm abnormalities (%) were recorded in control and(L_1) (2.00, 2.00 ± 0.25 %, respectively) atT_{48}, while the low value was recorded in control (1.00, 1.00 ± 0.25 %) at $T_0$and T_{24} throughout cooling period (T_0-T_{48})as illustrated in Table (2). Overall mean value of secondary sperm abnormalities (%) was significantly lower (P<0.05) in L_1 (4.66±0.85%) compared with L_2 or L_3 (6.22, 8.22±0.85%)respectively, while the higher (P<0.05) value was recorded in control (18.00 ± 0.85 %).In the meantime, the lowest (P<0.05) values was recorded in L_2 or L_3 compared to all treated specimens at T_0, while the highest value was recorded in control specimens (29.33±1.48 %) in T_{48} of chilled preservation at 4°C(table 2).

Regarding sperm acrosome integrity (%), the results illustrated that both L_2 and L_1 of vitamin E increased (P<0.05)(92.00, 91.55 ± 0.64 % respectively), followed by L_3 (89.00±0.64 %) and control (87.44±0.64 %) over time of storage. Furthermore, the higher (P<0.05) values of acrosome integrity (%) were recorded in L_2 and L_3, at time T_0hrwhile the lower value was recorded in control at $T_{48}$of chilled preservation at 4°C.Mean value percent of hypo-osmotic swelling test (HOST, %), were increased (P<0.05)in all Vit E supplemented specimens compared with control. Additionally, the higher (P<0.05) values of hypo-osmotic swelling test percent (%) were recorded in L_2 and L_3 at T_0while the lower (P<0.05) value was recorded in control at T_{48}ofchilledpreservation at 4°C(table 2).

Table.2: Effect of adding different concentrations of vitamin E in the diluent on physical characteristics of ram semen during 48 h of coldstorage at 4°C (Mean ± SEM)

Semen characteristics	Storage time (hr)	Treatment				
		Control	Levels of vitamin E			
			L_1	L_2	L_3	±SE
Sperm motility (%)	T_0	90.00^{c}	95.66^{a}	98.66^{a}	98.66^{a}	1.61
	T_{24}	85.00^{d}	87.33^{b}	94.20^{b}	81.66^{c}	
	T_{48}	82.00c	80.00^{c}	88.00^{b}	80.66^{c}	
Overall		85.66^{C}	87.66^{B}	92.22^{A}	87.00^{B}	0.93
Normal spermatozoa (%)	T_0	91.00^{b}	95.33^{a}	96.66^{a}	98.00^{a}	1.00
	T_{24}	81.00^{e}	95.33^{a}	91.33^{b}	88.00^{c}	
	T_{48}	66.00^{f}	90.66^{bc}	88.66^{bc}	85.00^{d}	
Overall		79.88^{C}	93.77^{A}	92.22^{A}	90.33^{B}	0.57
Primary sperm abnormalities (%)	T_0	1.00^{b}	1.33^{ab}	1.66^{ab}	1.00^{b}	0.25
	T_{24}	1.00^{b}	1.33^{ab}	1.33^{ab}	1.66^{ab}	
	T_{48}	2.00^{a}	2.00^{a}	1.66^{ab}	1.66^{ab}	
Overall		1.33	1.55	1.55	1.44	0.14
Secondary sperm abnormality (%)	T_0	7.33^{de}	3.33^{ef}	1.66^{f}	1.00^{f}	1.48
	T_{24}	17.33^{b}	3.33^{ef}	7.33^{de}	10.33^{cd}	
	T_{48}	29.33^{a}	7.33^{de}	9.66^{cd}	13.00^{cd}	
Overall		18.00^{A}	4.66^{C}	6.22^{CB}	8.22^{B}	0.85
Acrosome integrity (%)	T_0	94.00^{cd}	97.66^{ab}	98.00^{a}	98.00^{a}	1.10
	T_{24}	92.00^{cd}	95.66^{ac}	90.00^{de}	88.00^{e}	
	T_{48}	75.00^{f}	81.33^{f}	87.00^{e}	82.00^{f}	
Overall		87.44^{C}	91.55^{A}	92.00^{A}	89.66^{B}	0.64
HOST (%)	T_0	73.00^{e}	87.66^{ab}	91.33^{a}	91.33^{a}	1.66
	T_{24}	66.00^{f}	80.66^{cd}	80.66^{cd}	83.00^{bc}	
	T_{48}	46.00^{g}	82.00^{c}	76.66^{de}	75.00^{e}	
Overall		61.77^{B}	83.44^{A}	82.88^{A}	83.11^{A}	0.95

$^{a-f}$ values within the same row with different letters differ significantly ($P \leq 0.05$).

$^{A, B}$ values within the same column with different letters differ significantly ($p \leq 0.05$).

Effect of different levels of Vitamin E on oxidative status during preservation.

Changes in seminal plasma biomarkers and enzymes activities are presented in Figure (1).

Value of glutamic oxaloacetic transaminase (GOT, IU/L), was higher($P<0.05$) in the L_1 (270.62±8.61 IU/L) at T_{48} of chilled preservation at 4ºC, while the low ($P<0.05$) values were recorded in L_2 and L_1 (217.79,220.40±8.61 IU/L, respectively) at $T_0$of chilled preservation period compared with control (239.00 ±8.61 IU/L).The results also showed a severe decline ($P<0.05$) in glutamic pyruvic transaminase (GPT, IU/L) level in all Vit E- supplemented specimens over storage period compared with control (Fig. 1). Value of alkaline phosphatase (AKP, IU/L), was higher ($P<0.05$)in the L_3 (398.94±52.13 IU/L) at time T_{48}, while the low values were recorded in L_1 (60.69±3.65 IU/L) at T_0ofpreservationperiod.At T_{48} value oflipid peroxide (LP, nM/mL), was lower($P<0.05$) in L_2 (15.73±3.65 nM/mL) compared with L_1, L_3 (24.85, 26.16 ±2.63nM/mL, respectively) and control (24.85±2.63nM/mL). Value of hydrogen peroxidase (HP nM/mL), was higher ($P<0.05$)in the control specimens compared with differentlyolsoVit E-supplemented specimens over storage period (Fig. 1).

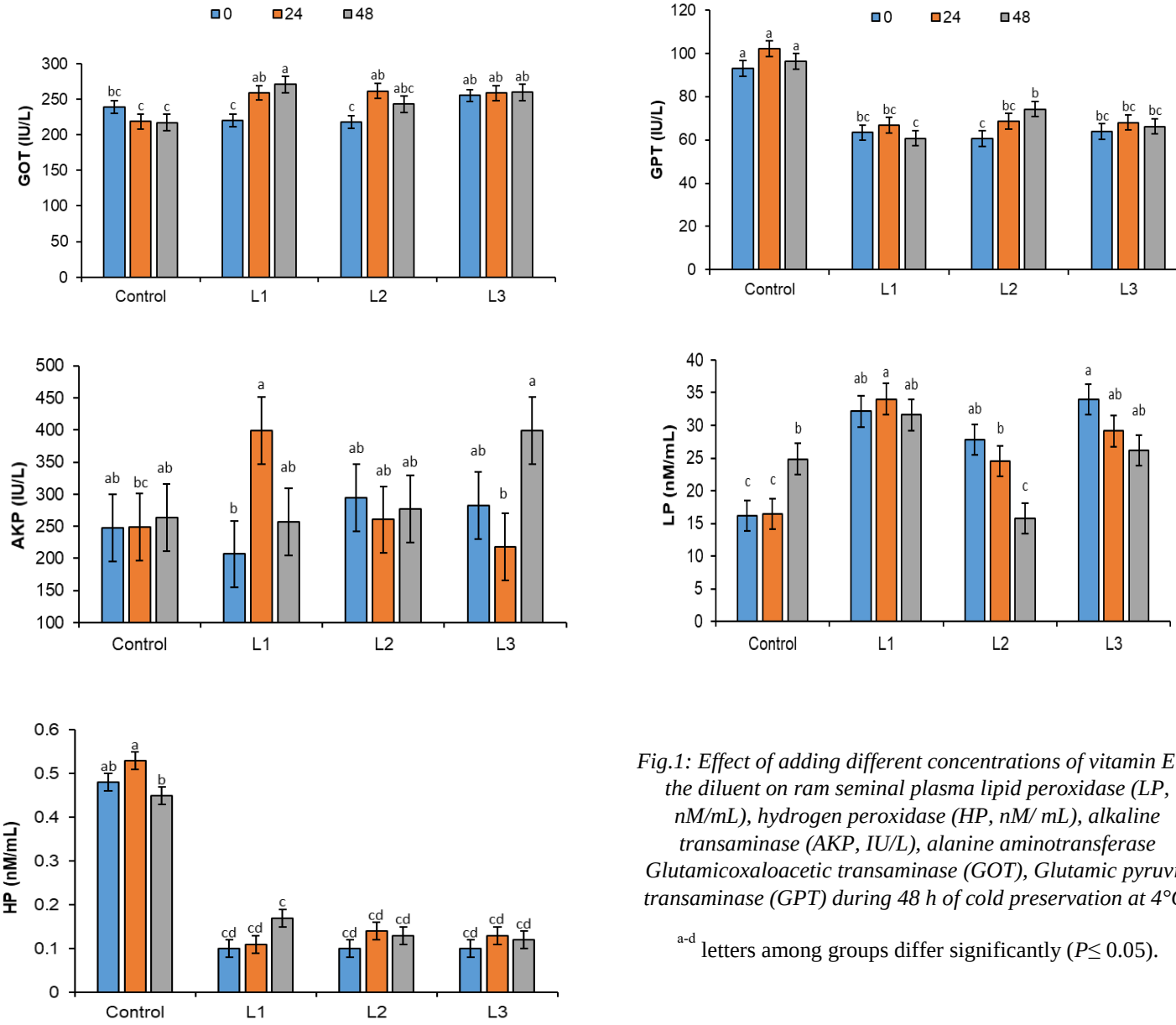

Fig.1: Effect of adding different concentrations of vitamin E in the diluent on ram seminal plasma lipid peroxidase (LP, nM/mL), hydrogen peroxidase (HP, nM/ mL), alkaline transaminase (AKP, IU/L), alanine aminotransferase Glutamicoxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) during 48 h of cold preservation at 4°C.

$^{a-d}$ letters among groups differ significantly ($P \leq 0.05$).

Table.3: Effect of adding different concentrations of vitamin C in the diluent on physical characteristics of ram semen during 48 h of cold storage at 4°C (Mean±SEM)

Semen characteristics	Storage time (h)	Treatment				
		Control	Levels of vitamin C			
			L_1	L_2	L_3	±SE
Sperm motility (%)	T_0	90.00^{e}	98.00^{a}	99.33^{a}	97.33^{a}	1.59
	T_{24}	85.66^{f}	84.00^{b}	89.33^{b}	85.33^{bcd}	
	T_{48}	82.00^{de}	77.66^{c}	88.00^{bc}	77.66^{e}	
Overall		85.66^{C}	86.55^{B}	92.22^{A}	86.77^{B}	0.93
Normal spermatozoa (%)	T_0	91.66^{b}	98.66^{a}	97.00^{a}	98.66^{a}	1.05
	T_{24}	81.66^{d}	91.33^{b}	91.33^{b}	88.66^{b}	
	T_{48}	66.33f	90.00^{b}	88.66^{b}	85.33^{c}	
Overall		79.88^{C}	93.33^{A}	92.33^{A}	90.88^{B}	0.61
Primary sperm abnormalities (%)	T_0	1.00^{bc}	1.00^{bc}	1.00^{bc}	0.66^{c}	0.27
	T_{24}	1.00^{bc}	2.00^{a}	1.33^{abc}	1.66^{ab}	
	T_{48}	2.00^{a}	2.00^{a}	1.66^{ab}	1.66^{ab}	
Overall		1.33	1.66	1.55	1.33	0.15
Secondary sperm abnormality (%)	T_0	7.33^{d}	1.00^{e}	2.00^{c}	1.00^{e}	1.52
	T_{24}	17.33^{b}	6.00^{d}	7.33^{d}	9.00^{cd}	
	T_{48}	29.33^{a}	8.00^{d}	9.66^{cd}	13.00^{bc}	
Overall		18.00^{A}	5.00^{C}	6.33^{BC}	7.77^{B}	0.88
Acrosome integrity (%)	T_0	94.33^{bc}	98.66^{a}	95.33^{bc}	97.00^{ab}	1.03
	T_{24}	92.33^{cd}	96.66^{ab}	90.66^{de}	87.66^{e}	
	T_{48}	75.66^{e}	81.33^{e}	87.33^{e}	82.00^{e}	
Overall		87.44^{B}	92.22^{A}	91.11^{A}	88.88^{B}	0.59
HOST (%)	T_0	73.33^{e}	95.33^{a}	92.00^{ab}	85.00^{cd}	1.68
	T_{24}	66.00^{e}	80.00^{de}	88.66^{bc}	80.66^{de}	
	T_{48}	46.00^{f}	82.00^{d}	76.66^{e}	73.33^{e}	
Overall		61.77^{C}	85.77^{A}	85.77^{A}	79.66^{B}	0.97

$^{a-f}$ values within the same row with different letters differ significantly ($P \leq 0.05$).

$^{A, B}$ values within the same column with different letters differ significantly ($P \leq 0.05$).

Effect of different levels of Vitamin C on chilled semen traits.

Means values ± SEM of sperm physical characteristics including sperm motility (%), normal sperm (%), sperm abnormalities (primary and secondary, %) acrosome integrity (%) and hypo-osmotic swelling test (HOST, %) are presented in table (3).

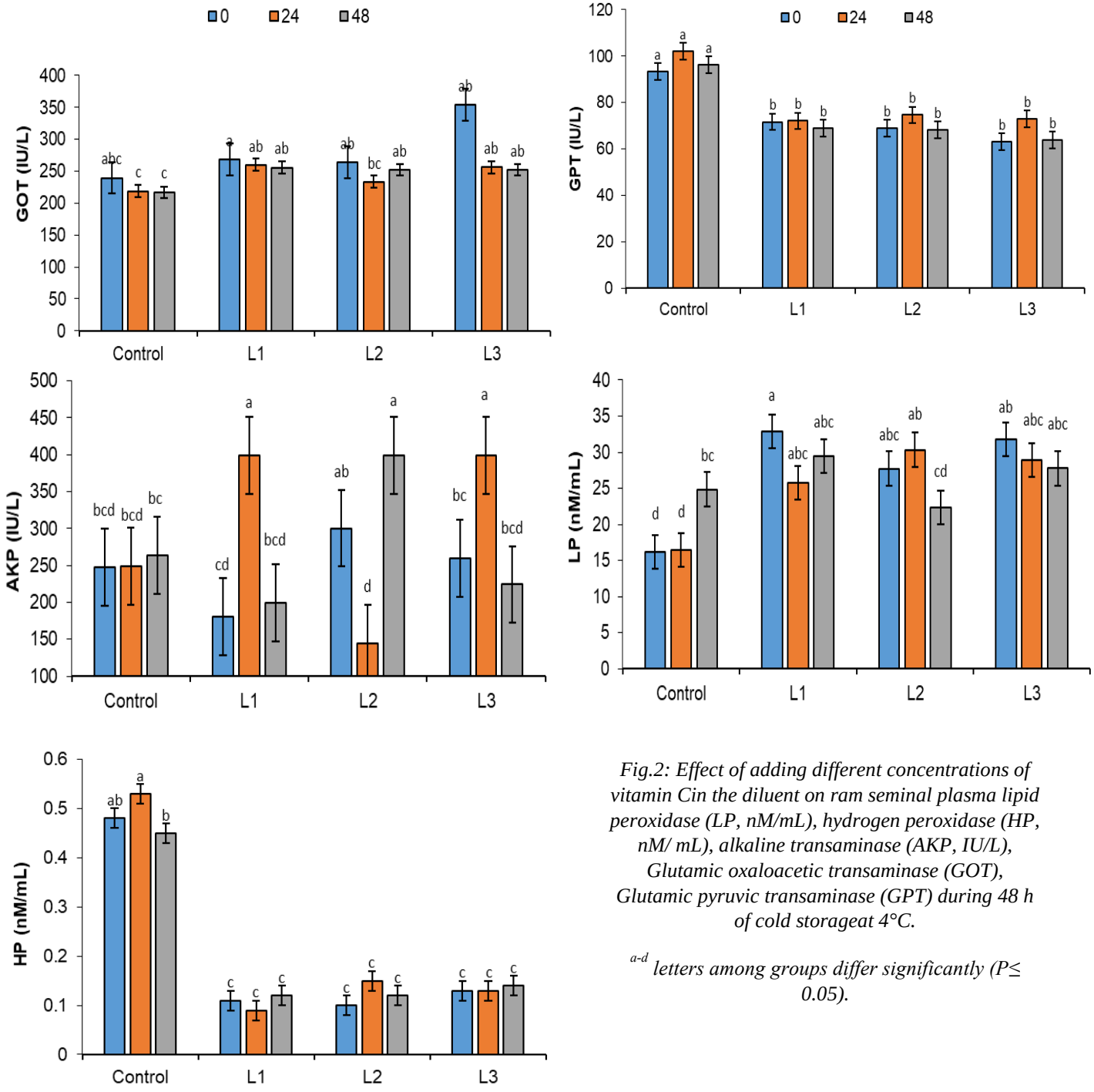

Fig.2: Effect of adding different concentrations of vitamin Cin the diluent on ram seminal plasma lipid peroxidase (LP, nM/mL), hydrogen peroxidase (HP, nM/ mL), alkaline transaminase (AKP, IU/L), Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) during 48 h of cold storageat 4°C.

$^{a-d}$ letters among groups differ significantly (P≤ 0.05).

Overall mean of sperm motility percent (%) was significantly higher (P<0.05) in L_2 of vitamin C (92.22 ± 0.93 %) compared with control (85.66±0.93%).Overall mean of normal sperm (%) was significantly higher (P<0.05) in (L_1) and (L_2) of vitamin C (93.33, 92.33 ± 0.61% respectively) compared with (L_3) (90.88±0.61%) or control (79.88±0.61%) at 48 hr of chilled preservation at 4°C. Contrarily, the lower (P<0.05) percent of normal sperm was recorded in control group at T_{48} of chilled preservation (Table 3). No significant difference was found in mean percent of primary sperm abnormalities (Table 3). There was interaction between time and treatment was recorded. The

higher (P<0.05) percent (%) was recorded in control and L_1, while the lower percent was recorded in L_2 and L_3at 48 hr of chilled preservation at 4ºC (Table 3). Overall mean of secondary sperm abnormalities percent (%) was significantly higher (P<0.05) in control (18.00 ± 0.88 %) followed by (L_3) (7.77±0.88%) and L_2 (6.33±0.88%) compared with L_1 (5.00±0.88%) during preservation period at 4ºC (Table 3). Also, the highest (P<0.05) percent was recorded in control group at T_{48} of chilled preservation at 4ºC (Table 3).

Overall mean value of acrosome integrity percent (%) was significantly higher (P<0.05) in both (L_1) and (L_2) groups of vit C, while the lowest values (P<0.05)were recorded in control and L_3 (87.44, 88.88 ±1.03 %) at 48 hr of chilled preservation (Table 3). A significant (P<0.05) interaction was noted between time and treatment. All levels of Vit C-supplemented or control specimens over storage period at T_0 and gradualdecline thought preservation period at T_{48} of chilled preservation 4ºC Table (3). Mean value of hypo-osmotic swelling test (HOST, %) was significantly higher (P<0.05) in L_1 and L_2 (85.77, 85.77 ± 0.97 % respectively)compared with control (61.77±0.97 %). The interaction between time and levels were significant (P<0.05), the highest value was recorded in L_1 (95.33±1.68 %) at T_0, while the lowest (P<0.05) value was recorded in control (46.00±1.68%) atT_{48} of chilled preservation at 4ºC (Table 3).

Effect of different levels of Vitamin C on oxidative status during preservation.

The changes in seminal plasma biomarkers and enzymes activities are presented in Figure (2).

At T_{48} of chilled preservation, value of glutamic oxaloacetic transaminase (GOT, IU/L), was higher (P<0.05)in all different levels of Vit C- supplemented specimens compared with control specimens. On the other hand value of glutamic pyruvic transaminase (GPT, IU/L), was increased(P<0.05) in the control (96.34±5.19 IU/L) compared with all treated semen specimens L_1, L_2 and L_3 (68.93, 68.13, 63.90 ± 5.19 IU/L), respectively (Fig. 2). In addition, value of alkaline phosphatase (AKP, IU/L), was higher (P<0.05)in the L_1 and L_3 at T_{48} respectively, while the low (P<0.05) values were recorded in L_1 at T_0 of preservation (Fig, 2). Value of lipid peroxide (LP, nM/mL), was lower (P<0.05) in the L_1 (22.36±2.32 nM/mL) at $T_{48}$of preservation at 4ºC, compared withsemen supplemented with other levels or control (Fig. 2). At T_{48} of preservation period,value of hydrogen peroxidase (HP nM/mL), was higher (P<0.05)in the control (0.45±0.02 nM/mL), while the lowest(P<0.05) values were recorded in all levels of vit C (Fig. 2).

IV. DISCUSSION

The results of the present work showed a significant enhancement in semen viability criteria in terms of sperm motility, normal sperm, sperm abnormalities (primary and secondary), acrosome integrity and HOST-reacted spermatozoa percentages in specimens supplemented with ascorbic acid or α-tocopherol. This finding is in agreement with previous studiesin different species (Abd El-Hamid *et al*., 2018; Foote *et al*., 2002; Yoshimoto *et al*., 2008; Mirzoyan *et al*., 2006), where addition of both vitamins efficiently improved physical characteristics and functional integrity of cold-stored or frozen spermatozoa. Vitamin E is one of the vitamins that cannot be synthesized by mammalian cells. Nonetheless, it hasbeen reported to play an important role in the antioxidant defense system against ROS and LPO in the sperm cell (Almeida and Ball, 2005). Such ability was attributed to its capacity to inhibit LPO reaction in cell membranes by eliminating peroxyl (ROO•), alkoxyl (RO•), and other lipid-derived radicals (Silva, 2006). Moreover,it has been reported responsible forreducingSOD and LP in sperm cell membrane during cooling stress (Andrabi *et al*., 2008; Zhang *et al*., 2001). Vitamin C, on the other hand, is considered the major non-enzymatic electron-trapping component, and playsa major role in trapping molecules in the seminal fluid (Zeitoun and Al-Damegh, 2015), thus, reducing the levels of reactive oxygen species (Fernandez-Santos *et al*., 2009; Hu *et al*., 2010). Additionally, it improves sperm metabolic activity and viability, as well as mitochondrial membrane potential in sperm cells (Hu *et al*., 2010)in the present work, Vitamin C supplementation led to decrease in pH due to its strongly acidic properties (10% solution: pH 2). Similar finding has been previously reported to induce reversible or irreversible reductions in motility (Acott and Carr, 1984).

In the present study the enzyme activities ofGOT in specimens supplemented with both additives increased at 48 h of preservation at 4ºCalongside AKP activity. This agrees with previous observations of Zeiton& Mona(2015), and may directly indicate sperm membrane damage (Pesch*et al*., 2006), and increased percentages of dead and abnormal spermatozoa (Gundogan*et al*., 2010).

Contrariwise, the results showed that values of LP, HP and GPT concentrations declined in specimens

supplemented with different levels of vit. E or C at 48 h of preservation at 4ºC.This could be attributed to actions of ascorbic acid and α-tocopherol on the membrane structure in sperm cell, particularly when added to semen extenders in higher doses (AbdEl-Hamid *et al*., 2018; Srivastava and Kumar, 2014;Gundogan *et al*., 2010). In smaller concentrations, however, ascorbic acid through its ROS-reducing properties (Ball *et al*., 2008) might have minimized the probable oxidative damages to the membranes,thereby preventing the enzyme leakage (Amidi*et al*., 2016; Kheradmand*et al*., 2006; Chatterjee and Gagnon 2001).

V. CONCLUSIONS

The results indicated that addition of adequate concentrations of ascorbic acid or α-tocopherol to semen diluent can reduce the possible damage induced by oxidative stress, hence, maintain physical characteristics of ram spermatozoa during 48 h of cold storage. Nevertheless, inclusion of the optimum levels of both vitamins combined in the diluent may further improve the fertilization potential of spermatozoa prior to application of artificial insemination (AI) and in vitro fertilization (IVF) in sheep.

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Organic Treatment effects on Ferritic soil quality and Tomato (*Lycopersicon esculentum* Mill.) Yield

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***Abstract*— *The impact of the combination of plant (Tithonia diversifolia) (Td) plus cow dung (Cd) as biofertilizer and aqueous extract of Callistemon citrinus (CAL) leaves as biofungicide on physicochemical properties, and the microbial biomass in carbon (MBC) and nitrogen (MBN) of soil and on tomato yield were assessed under field condition. The experimental design was a complete block design with 2 factors (soil amendment and plant sprays) and 3 repetitions. The soil treatment included organic amendment (OA): Td + Cd at the ratio of 3:4 (w/w)/plant; inorganic amendment (IA): 21:8:8 NPK (26.2g/plant) and potassium sulfate (4g/plant); and control (unamended soil). The field treatments were plants sprayed with: 5% (w/v) CAL; 5% (w/v) Mancozeb (M); and water (W). All amendments except IA did not significantly modify the soil organic matter (<2.4mg.kg^{-1}) and organic carbon content. An increase of 23.15% and 30.60% of calcium concentration and cation exchangeable capacity (CEC) respectively, was recorded in OA soil compared to the soil before cultivation (SBC) (P<0.05). Copper and zinc contents in OA soil were reduced respectively by 49% and 48.5% compared to SBC. The highest concentration of MBC was recorded in OA.M plot. The different combine treatments (OA.CAL, IA.M, and OA.M) increased tomato yield by 3.4; 3; and 5.3 fold, respectively compared to their controls. This study provided new information about the organic amendment on soil and plant sprayed with C. citrinus extract as a green alternative to conventional input that might improve soil quality and crop yield.*

Keywords*— *microbial biomass, organic inputs, soil physicochemical properties, tomato.

I. INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is among the most important fruit vegetables in the world due to the high potential health-promoting properties and their contents in flavonoids, carotenoids, and vitamins (Aghofack-Nguemezi and Schawb, 2014). Tomato yield and production were 188t/ha, 182301395tons and 12.1t/ha, 1279853 tons respectively in the world and Cameroon (FAOSTAT, 2019). Pests and diseases are among the most important constraints of tomato culture in Cameroon. The farmers currently apply conventional agrochemicals in an abusive and inappropriate manner (Konje et al. 2019). The use of pesticides and their residues poses a real public health problem to farmers, consumers and the environment (Zinkakuba et al. 2019). It is therefore urgent to search for alternative methods that are environmentally friendly and with fewer health risks to the consumers.

The disease control of crops through the use of plant-based biopesticides and antagonistic microorganisms has become an interesting alternative (Olanya and Larkin, 2006). In Cameroon, local plant extracts have been reported as having inhibitory properties *in vitro* and *in vivo* against various pathogens (Goufo et al. 2010; Ndonkeu et al. 2013; Mekam et al. 2019). Botanicals with antifungal compounds have been identified and can be exploited for the management of plant diseases because they have low mammalian toxicity, target specificity, biodegradability and contain many active ingredients (Kagale et al. 2004). *Callistemon citrinus* because of its antifungal activity notably against *Phytophtora infestans* of tomato (Galani et al. 2013; Dakole

et al. 2016), on *Alternaria padwickii* and *Bipolaris oryzae* of rice (Nguefack et al. 2013) is used as a biocontrol agent.

To achieve high tomato yield, farmers used chemical fertilizers, which could be responsible for soil acidification and the decrease in the microbiological activity of soils (Monkiedje et al. 2006). Also, the use of agrochemicals causes the long-term accumulation of heavy metals in water and soil. These heavy metals are assimilated by plants and enter the food chain causing animal and human health problems (Zwolak et al. 2019). As well, the rising costs of inorganic fertilizers made them too expensive.

Tithonia diversifolia (Asteracea) green biomass is an effective source of nutrients and has been used successfully to improve soil fertility and crop yields in Kenya (Jama et al. 2000). Aguyoh et al. (2010) reported a significant and positively correlated increase in a total yield of watermelon with increasing application rates of *T. diversifolia* manure. In that study, *T. diversifolia* application enhanced the total yield of watermelon by between 8.5% and 31% compared to the control. *T. diversifolia* can improve the physical and chemical properties of soil and increase nutrients in the soil (Crespo et al. 2011) and maintain soil fertility for a long period (Babajide et al. 2008).

Cattle manure used for its added value for soil carbon sequestration, and it's capacity for storing and releasing nutrients over a longer period (Diacono and Montemurro, 2010). In Uganda, although a significant number of farmers have adopted the use of cattle manure on their farms, they normally use it untreated and directly from animal barns (Komakech et al. 2014). According to Adegunloye et al. (2007), the C: N ratio in cow dung manure is an indication that it could be a good source of protein for the microbes involved in the decomposition of organic matter.

Each amendment applied to the soil may not contain all required nutrients in a high amount at the same time. Therefore, to have a balanced nutrient supply, the addition of more than one amendment to the soil may be required. Adekiya (2018). Consequently, in this work, it was planned to test a biofertilizer made of a mixture of Td + Cd. The present study aimed to evaluate the impact of the combination of plant (*Tithonia diversifolia*) (Td) plus cow dung (Cd) as biofertilizer and aqueous extract of *Callistemon citrinus* (CAL) leaves as biofungicide on physicochemical properties and the microbial biomass in carbon (MBC) and nitrogen (MBN) of soil and on tomato yield in field condition.

II. MATERIAL AND METHODS

1.1 Experimental site

The experiment was conducted at Nkolbisson-Yaounde (Eloundem) Centre Region of Cameroon from December to May 2018. The study site is located at an altitude of 711m above sea level and situated at latitude 3°51'14"N and longitude 11°44'26"E. The annual rainfall distribution is bimodal (lighter rains between March and June and a more intense rainy season between September and November) with peak rainfall in May and October. The area has a mean annual rainfall of approximately 1500-2000mm and a mean annual temperature of 24.7°C. The relative humidity range between 50 and 80% in the dry season and 70 and 90% in the rainy season. The most dominant soil types at Nkolbisson is ferritic and acidic (pH 5- 6).

1.2 Field design

The variety of tomato (Cobra) seeds used was produced by the French firm TECHNISEM. Tomato seeds were sown in nursery beds enriched with the mixture of *Tithonia diversifolia* (*Td*) leaves and cow dung (Cd) powders at the dose of 312.5 and 250 g/m^2, respectively. The tomato plants used were 35 days old and had three to four true fully expending leaves.

The field experiment was laid out in a complete block design, with 9 plots per block and three repetitions. Each plot contained 16 plants and made up of 4 rows. Tomato seedling transplanting was done at an interval of 0.5m between rows and 0.5m between plants of the row. In each plot, 16 pockets were dug and each filled with organic amendment (*Tithonia diversifolia* leaves (75g/plant) and cow dung (100g/plant) powders) one week before transplanting. In the inorganic plots, 21:8:8 N/P/K (26.2g/plant) was applied one week after transplanting and potassium sulfate (50%K_2O and 45% SO_3) (4g/plant) at fruit set stage. Organic and inorganic nitrogen was applied an equivalent of 5g of N/plant. Plants were sprayed 10 times (twice/month) with water, 5% of both biopesticide, and chemical pesticide. The biopesticide was obtained by soaking the powder of dried leaves of *Callistemon citrinus* in water for 24 hours. The chemical fungicide was mancozeb.

1.3 Soil sampling

In each plot, the soil was sampled at a depth of 0-20 cm in three different areas diagonally. All sample soils were mixed to form a composite. 0.5 kg of the composite was air-dried, ground, and sieved (<0.25 mm). Then serve as a substrate for the physicochemical analysis. A part of the composite was sieved (<0.5 mm) and then stored at 4°C until microbial biomass analyses.

2.4 Physicochemical analyses

Soil properties were observed both before and at the end of the experiment. Granulometry was determined by the

Robinson-Köhn pipette method. Soil pH was measured in 1:2.5 soil to solution ratio in distilled water (pH-H20). Organic carbon (OC) was estimated by oxidation with potassium dichromate and titration with ferrous sulfate (Walkley and Black, 1934). Total nitrogen was estimated by the Kjeldahl method. Iron, copper, lead, and zinc were determined colorimetrically after reduction with dithionate-citrate-bicarbonate (DCB). Available phosphorus was determined by the Bray II method (Bray and Kurtz, 1945). Calcium was estimated by a complexometric and titrimetric method with tetra acetic ethylene diamine acid of an ammonium acetate extract at pH 7 of the sample. Cation exchange capacity (CEC) was determined by percolating 2.5g of soil with 100mL of 1N ammonium acetate buffered at pH 7, removing the excess with ethanol and displacing the absorb NH_4^+ ions with 1N KCl, determining the collected NH_4^+ ions by distillation and titration with 0.01N sulfuric acid.

2.5 Study of soil microflora

Microbial biomass was determined by fumigation-extraction (Chaussod et al. 1988; Wu et al. 1990). Carbon determination was done by Walkley and Black (1934) and nitrogen determination by Kjeldahl. The coefficients used to determine the biomasses were: KeC = 0.38 and KeN = 0.68.

2.6 Tomato yield

From each plot, matured tomato fruits were harvested each week and total fruit weight was determined. Fruit product data were summed up of the total fruit weight from consecutive harvests and converted into tons per hectare to estimate the fruit yield.

2.7 Statistical analysis of data

The results were subjected to statistical analysis using IBM SPSS Statistics 22 software, particularly variance analysis (ANOVA) and significant differences were assessed using the Student Newman Keuls (SNK) test at the 5% probability threshold. XLSTAT 2007 software was used for the Principal Component Analysis (PCA).

III. RESULTS AND DISCUSSION

3.1 Effects of organic amendment on soil physicochemical properties

The effect of different amendments on soil granulometry showed some variations in particle contents (Table1). The silt content was above 45%, and double that of sand. Clay and sand contents from soil samples collected before cultivation (SBC) and control soil, were significantly ($P<0.05$) lower, compared to those of organic amended (OA) and inorganic amended (IA) soils. Based on the USDA textural diagram, the texture observed in SBC and control soil was silty clay loam and clay loam in OA and IA soils. Silt clay texture was favourable for tomato cultivation.

The carbon-nitrogen ratio (C:N) was found to be 8 in OA soil combined to plant treated with the aqueous extract of *C. citrinus* (OA.CAL). Also, C:N was less than 14 in SBC, IA soil, OA soil combined to plant treated with mancozeb (OA.M) and 16.75 on the control soil combined to plant treated with water (Control.W) (Table 2). IA.CAL soil (C:N = 8) with the same soil organic matter (SOM) content as SBC soil (C:N = 11) had higher total nitrogen (TN) concentration. Hubert and Schaub (2011) showed that high carbon-nitrogen (C:N ≥13.70) made decomposition slow to difficult and did not allow good mineralization of organic matter. Soils, where plants have been sprayed with water, had the lowest amounts of available phosphorus (P) ($P<0.05$). This could be explained by the fixation of available phosphorus in these soils by protons and cations such as Ca^{++} and thus transforming it into a compound not assimilable by the plant (Rivaie et al. 2008). The highest P content (15.64mg.kg-1) of about 2 fold that of SBC ($P<0.05$) was recorded in OA.CAL soil. Similar content in SOM (2%) was recorded in all treated soils except for IA.M (2.92%) and IA.W (3.75%) soils ($P<0.05$). The variation in SOM could be explained by the rapid supply of IA soil by chemical fertilizer. Beside, Dieye et al. (2016), showed that the mineralization of *T. diversifolia* was slow and progressive. The 26% reduction of TN OA soil compared to SBC, could be explained by high C:N (14) observed in OA soil. Bationo et al. 2008 suggested that rapid decomposition of SOM could result in loss of nutrients through volatilization, leaching; whereas, for slow mineralization of SOM, minerals will have a higher retention time in the soil.

The TN content increased respectively, in IA.M (1.31g/kg), IA.CAL, and IA.W soils where it reached 1.92g/kg. These results differed from those obtained by Hafifah et al. (2016), where *T. diversifolia* and cow manure mixture was significantly enriched in OC and TN than the NPK. This could be explained by the high levels of fertilizer used in their study of 1.35 t/ha NPK, 4.08 t/ha *T. diversifolia* and 12.93 t/ha cow manure; the type of crop (cauliflower) and the date of collection of soil samples at the end of the cultivation, which was 30 days. The Control.W soil had the lowest nitrogen content, 1.67 times less than in the SBC. The nitrogen decrease in control soil could be explained by the use of existing nitrogen in the soil for the development of soil microorganisms and even the growth of tomato plants. These results corroborate those of Hafifah et al. (2016), who recorded a 1.83-fold decrease in the total nitrogen content of the control soil compared to the initial soil when the cauliflower was grown.

Calcium (Ca) concentration and cationic exchange capacity (CEC) were similar in OA and IA soils and were higher than in SBC and control soils (P<0.05) (Table 3). This increase could be explained by their relatively high clay content, able to bind to organic matter to form the humic argil adsorbent complex. The latter plays an important role in the cation exchange capacity (CEC) for storing many nutrients in the soil and the water retention capacity or Useful Reserve Hubert and Schaub (2011). Also, Ca^{2+} stabilizes the adsorbing complex by creating a calcium bridge that consolidates the connection between humus and clay. The pH values of the different soils were similar (P<0.05) and varied between 6.52 and 7.05. The pH did not vary during the culture and remained neutral. The soil pH, less than 6.0 tends to be acidic with very high exchangeable aluminum that restricts the growth of most crops (Fairhurst, 2012). Copper (Cu) concentration was reduced by 46.1, 50.7 and 51.9% respectively, in OA.CAL, OA. M, and OA.W soils compared to SBC with the highest Cu concentration (20.53mg.kg^{-1}) (P<0.05). This decrease could be explained either by the chelation and precipitation of copper by organic matter present in these soils or by the use of copper by the plant for the maturity of fruits (Lopéz-Vargas et al. 2018). All IA soils had a similar Cu content but showed a reduction of 41% compared to SBC soil (P<0.05). The increase of Cu in IA.M soil compared to OA.M soil could be due to the supply of these soils by chemical inputs. The iron (Fe) concentration of SBC was identical to that of IA soil and with a decrease of 7.4 and 4.42% respectively, in OA.W and OA.M soils (P<0.05). The concentration of Zn varied according to the nature of sprayed products. The Zn lowest concentration was recorded in OA soils (18.8mg.kg^{-1}), representing 48.5% decrease compared to SBC. This could be due to the complexation of free Zn with organic matter (Angelova et al. 2013). In control soil, mancozeb further increased the soil Zn concentration followed by CAL extract (P<0.05). The further increase of Zn to mancozeb plots could be attributed to its Zn as component chemical composition. In general, there was no accumulation of heavy metals in OA soil. This phenomenon could prevent the long-term onset of soil toxicity.

3.2 Effects of organic amendment on soil microbial biomass

Microbial biomass in carbon (MBC) was enriched in OA and IA soils than SBC and control soil (P<0.05) (Table 4). These amended soil also had high clay content. In 2007, Kasel and Bennett reported that an increase in soil-clay increases soil micropores hence limiting the development of microorganism predators and thus a protective effect on total microbial biomass. The highest MBC value was recorded from OA.M soil with an increase of 5.8% and 42.65%, as compared respectively to IA.M soil, and SBC (P<0.05). High soil microbial biomass often leads to high nutrient availability to crops thus enhancing both the microbial biomass turnover and the degradation of non-microbial organic materials (Tu et al. 2006). In the amended soil, MBC vary as a function of the nature of the sprayed products. The MBC content with respect to SBC (P<0.05) increased by 20.27% in IA.W and OA.W soils; 25.16% in OA.CAL soil; 34.3% in IA.CAL and IA.M soils; 42.64% in OA.M soil. The lowest values of microbial biomass in nitrogen (MBN) were recorded in OA.W soil (0.7mg N.kg^{-1}) and OA.CAL (0.8mg N.kg^{-1}). A five (5) % increase in MBN was observed in OA.M soil as compared to IA.M soil (P<0.05).

3.3 Effects of organic amendment on tomato yield

The tomato yield was a function of the amendment (Fig 1). The highest yields (107 to 7.33t/ha) were recorded from OA soils, followed by IA soils with yield values ranging from 60.2 to 8t/ha. Control soils had the lowest yields (P<0.05). Tomato yield of OA.M plot was 1.8 fold higher that of IA M plot (P<0.05). OA CAL plot increased tomato yield by 21% compared to IA CAL plot (P<0.05). Ghorbani et al. (2008) found that cattle manure in Iran did not give a good yield of tomato compared to the use of chemical fertilizers. The highest increase of tomato yield obtained with OA.M in this study might be explained by the application of green manures which reduced soil bulk and increased porosity, nutrient content (Adekiya, 2019). Easily available and excessive nitrogen fertilization from inorganic fertilizers delays maturity and may reduce tomato yield. Delayed maturity results in foliage exposed to potential infection for a longer time, increasing the risk of fruit diseases (Ghorbani et al. 2008). The yield increases with the nature of sprayed products; it was higher when the plants were treated with mancozeb fungicide, followed by CAL extract and finally water (P<0.05).

3.4 Main Component Analysis between some indicators of soil and different amendments

The correlation diagram (*Fig2*) showed that fertility indicators such as P, MBN, TN, and SOM contributed to the formation of the F2 factor and other fertility indicators contributed to the formation of the F1 factor. MBC was strongly correlated with total nitrogen with a correlation coefficient (r=0.801 to P <0.05). Partey et al. (2017), who reported that soil treatment with *T. diversifolia* recorded the greatest effect on the increase of mineral N, soil microbial biomass and β-glucosidase activities, obtained similar correlation results. The MBN was negatively correlated with P (r= -0.751 P <0.05). There were strong correlations between clay and Ca (r=0.865 P 0.05), clay and cation exchange capacity (r=0.845 P <0.05), with a very strong correlation between Ca and CEC (r=0.932). The yield was

correlated with the adsorbent complex (CEC, Ca, clay). Control soils were negatively correlated to tomato yield and the adsorbing complex. Houot and Chaussod (1995) found a positive correlation between microbial biomass and soil carbon content on wheat-beet rotation conducted with different fertilization types and levels. They also noted decreasing biomass values according to the types of fertilization in the following order: farm fertilizer>mineral fertilization> no fertilization.

IV. FIGURES AND TABLES

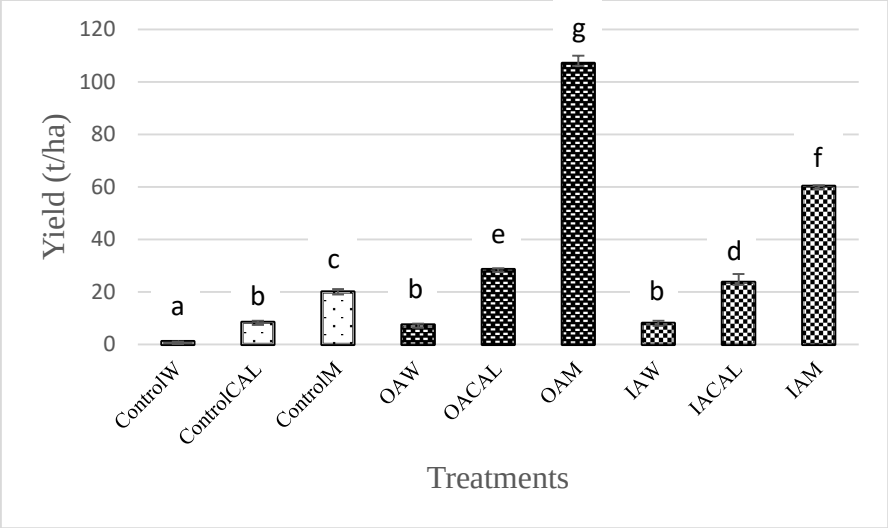

Fig 1: Tomato yield production

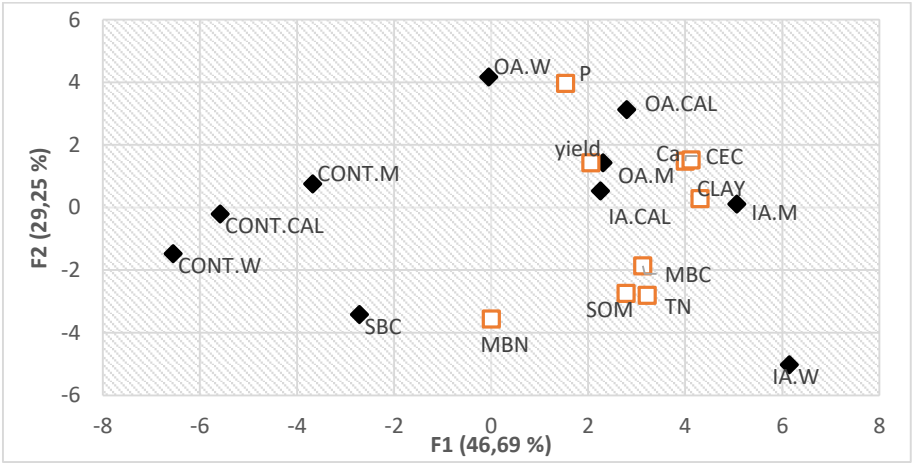

Fig 2: correlation diagram between some soil indicators and different amendments

Table 1: Granulometry of soil collected at the beginning and end of Lycopersicon esculentum field cultivation

Variables	Clay (%)	Silt (%)	Salt (%)
SBC	29.66^{a}	52^{d}	18.33^{a}
Control.W	29^{a}	51.66^{d}	19.26^{b}
Control.CAL	29^{a}	51.5^{d}	19.5^{bc}
Control.M	29.66^{a}	51.5^{d}	19.5^{bc}
OA.W	30.33^{ab}	48^{c}	21.5^{c}
OA.CAL	33.66^{c}	46.66^{b}	20.5^{c}
OA.M	32.33^{abc}	46.57^{b}	22.5^{d}
IA.W	33.33^{bc}	45^{a}	22^{d}
IA.CAL	31^{ab}	45.75^{ab}	23.5^{e}
IA.M	33.33^{bc}	45^{a}	21.75^{d}

Numbers followed by different letter notation in the same column are significantly different based on the Student Newman Keuls (SNK) test at the 5% level.

Table 2: Macroelements of soil collected at the beginning and end of Lycopersicon esculentum field cultivation

Variables	C:N	OC (%)	P ($mg.kg^{-1}$)	SOM (%)	TN ($g.kg^{-1}$)
SBC	11±1b	1.37±0.04bc	7.96±1.08c	2.36±0.28a	1.23±0.07d
Control.W	16.75±0.25e	1.24±0.01ab	5.95±0. 2ab	2.13±0a	0.74±0a
Control.CAL	14.75±0.75d	1.35±0.1bc	9.71±0.18d	2.3±0.17a	0.9±0bc
Control.M	13.75±0.25cd	1.24±0.01ab	10.53±0.26d	2.13±0a	0.9±0bc
OA.W	14.75±0.25d	1.25±0ab	4.14±0.33a	2.16±0.1a	0.86±0b
OA.CAL	14.25±0.75cd	1.39±0.08c	15.54±0.65f	2.39±0.14a	0.96±0.01b
OA.M	12.75±0.25c	1.28±0.03abc	10.76±0.05d	2.2±0.04a	0.99±0.01c
IA.W	11.5±0.5c	2.18±0.05e	6.57±0.22b	3.75±0.07c	1.92±0.05g
IA.CAL	8±1a	1.18±0.07a	11.79±0.47e	2.03±0.11a	1.44±0.03f
IA.M	13.25±1.25cd	1.69±0.05d	11.98±0.23e	2.92±0.16b	1.31±0.08e

Numbers followed by different letter notation in the same row are significantly different based on the Student Newman Keuls (SNK) test at the 5% level.

Table 3: Mineral elements of soil collected at the beginning and end of Lycopersicon esculentum field cultivation

Variables	SBC	Control.W	Control.CAL	Control.M	OA.W	OA.CAL	OA.M	IA.W	IA.CAL	IA.M
Ca^{++} ($cmol^{(+)}.kg^{-1}$)	12.18 ± 0.52^{b}	11.41 ± 0.22^{ab}	10.64 ± 0.34^{a}	12.47 ± 1.49^{b}	15.88 ± 0.12^{c}	16.07 ± 0.26^{c}	15.63 ± 0.1^{c}	15.77 ± 0.34^{c}	15.8 ± 0.01^{c}	15.81 ± 0^{c}
CEC ($cmol^{(+)}.kg^{-1}$)	30.81 ± 1.53^{a}	33.61 ± 1.13^{b}	34.3 ± 0.02^{b}	38.62 ± 3.37^{c}	43.5 ± 0.24^{d}	45.68 ± 0.89^{d}	44.1 ± 0.22^{d}	46.7 ± 0.76^{d}	46.52 ± 0^{d}	46.03 ± 0.63^{d}
pH	6.8 ± 0.62^{a}	6.55 ± 0.05^{a}	7.05 ± 0.05^{a}	6.75 ± 0.25^{a}	6.52 ± 0.02^{a}	6.62 ± 0.07^{a}	6.57 ± 0.02^{a}	6.67 ± 0.27^{a}	6.8 ± 0.15^{a}	6.8 ± 0.1^{a}
Cu ($mg.kg^{-1}$)	20.53 ± 0.38^{d}	11.64 ± 0.04^{c}	11.67 ± 0.17^{c}	12.03 ± 0.1^{c}	9.88 ± 0.18^{a}	11.06 ± 0.09^{b}	10.12 ± 0.18^{a}	12.04 ± 0.36^{c}	12.04 ± 0.09^{c}	12.08 ± 0.2^{c}
Fe ($mg.kg^{-1}$)	84.96 ± 0.78^{d}	83.8 ± 0.2^{d}	77 ± 0.1^{a}	80.72 ± 0.22^{c}	78.67 ± 1.27^{b}	84.64 ± 0.74^{d}	81.2 ± 1.7^{c}	83.08 ± 0.17^{d}	83.59 ± 0.63^{d}	83.7 ± 0.29^{d}
Zn ($mg.kg^{-1}$)	28.98 ± 0.69^{e}	19.89 ± 0^{b}	21.35 ± 0.29^{c}	22.94 ± 0.5^{d}	17.79 ± 0.14^{a}	19.35 ± 0.05^{b}	19.36 ± 0.56^{b}	21.95 ± 0.07^{c}	23.63 ± 0.01^{d}	22.9 ± 0.36^{d}

Numbers followed by different letter notation in the same row are significantly different based on the Student Newman Keuls (SNK) test at the 5% level

Table 4: microflora of soil collected at the beginning and end of Lycopersicon esculentum field cultivation

Variables	MBC ($mg.kg^{-1}$)	MBN ($mg.kg^{-1}$)
SBC	440.36 ± 0.93^{a}	0.9 ± 0^{e}
Control.W	455.37 ± 0.42^{a}	0.89 ± 0.01^{e}
Control.CAL	478.21 ± 5.95^{b}	0.82 ± 0^{c}
Control.M	462.88 ± 8.17^{a}	0.85 ± 0^{d}
OA.W	534.24 ± 5.64^{c}	0.7 ± 0^{a}
OA.CAL	551.17 ± 3.75^{d}	0.8 ± 0.01^{b}
OA.M	628.16 ± 5.63^{f}	0.89 ± 0.02^{e}
IA.W	524.85 ± 3.76^{c}	0.9 ± 0.01^{e}
IA.CAL	586.83 ± 3.1^{e}	0.85 ± 0.02^{d}
IA.M	596.31 ± 13.08^{e}	0.85 ± 0^{d}

Numbers followed by different letter notation in the same row are significantly different based on the Student Newman Keuls (SNK) test at the 5% level

V. CONCLUSION

From this study, it established that organic amendment combined with treatment of tomato plants with extract of Callistemon Citrinus improved soil quality by increasing available phosphorus, calcium, cation exchange capacity, and by reducing soil heavy metal accumulation. Therefore, it constitutes an environment eco-friendly integrated strategy for soil fertilization. Also, the combination of organic amendment and chemical pesticide (mancozeb) spraying improved MBC concentration and tomato yield. This combination could be a solution in crop production management. Whereas, low concentration of organic amendment did not increase SOM content and thus there need for further investigation to establish an optimum concentration of organic inputs.

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Performance, health status and cost implications of Raising Broiler chickens under different housing Systems

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***Abstract*— *This study investigated the performance and cost implication of raising broiler chickens under different housing systems. Three (3) different rearing systems namely; deep litter, colony cage and fold unit housing systems were used for this study. A total number of one hundred and thirty (130), four (4) weeks old Abor-acre strain of broiler chickens were procured out of which, one hundred and twenty (120) were randomly distributed into three (3) treatments of four (4) replicates, ten (10) started chicks per replicates in a Completely Randomized Design and the weight of each replicate was balanced (±1g). The birds were fed formulated broiler finisher diets and water ad–libitum throughout the experimental period. At the end of the experimental period, the birds were starved overnight and weighed in the morning. Two (2) birds per replicates were slaughtered and blood collected for haematological studies. Carcass and organ parameters were recorded, while cost analysis was done using excel. Data collected were subjected to analysis of variance using SPSS version 17 package. Results from the study revealed highest final weight, highest weight gain, highest eviscerated weight, lowest feed intake (2388.00 ± 1.44g, 1716.25 ± 0.32g, 1890.00 ± 27.00g, 3475.75 ± 36.57g, respectively) and best feed conversion ratio (2.03 ± 0.07) were recorded in birds on fold unit system. All the organs measured were significantly (P<0.05) influenced by the treatments except heart. Erythrocyte sedimentation rate (ESR), packed cell volume (PCV), heamoglobin (Hb), mean cell heamoglobin concentration (MCHC) were not significantly (P>0.05) influenced by the housing systems. Among all the bacteria isolated; streptococcus faecalis, Salmonella spp., Enterobacter aerogenes and Seratia marcesces were significantly (P<0.05) influenced by the housing systems. The cost evaluation of this study indicated beneficial effect of using colony and fold unit systems. Lowest total cost of production (₦1481.67) was recorded in fold unit system while highest total cost of production (₦1754.95) was recorded in deep litter system. Highest live weight (2.44kg), highest total sales price (₦2196.00) and highest net profit (₦714.00) were recorded in bird raised under fold unit. From the total cost of production, live weight, total sales price and net profit, it could be concluded that bird raised under fold unit performed well and better and such housing system (fold unit) could be adopted by backyard/small scale broiler chicken farmers as alternative to conventional deep litter system.*

Keywords*— *Production, cost, broiler chickens, housing systems, blood, bacteria.

I. INTRODUCTION

With the present economic situation in Nigeria and considering the rate at which an average Nigerian tends to raise chickens (broilers and cockerels) during Christmas and New year festivals in their compounds call for need to look for ways to encourage these backyard farmers in order to reduce the cost of poultry production and management with a view to encouraging livestock farmers to produce more animal proteins for the populace without compromising the quality of meat obtained from these animals. There is the need to develop more convenient, easily adoptable housing systems which are safer and cost effective for these backyard poultry farmers. Apart from the major argument about the welfare of poultry managed

and housed intensively, there is evidence that compromised welfare is usually associated with a reduction in productivity (Jones *et al*., 2005; Julian, 2005). Cost of conventional buildings; battery cages, deep litter system is now a great challenge as cost of building materials is going up on daily basis in Nigeria due to growing inflation and exchange rates, there is therefore the need for cheaper housing systems. An attractive hypothesis to consider is that put forward recently by (Dawkins *et al.*, 2004), which suggests that "chicken welfare is influenced more by housing conditions". This hypothesis therefore, should be expected to account also for the use of cages in the production of broiler chickens. Hence, the question that arises is how performance responses (growth characteristics and carcass quality) of broilers are related to different housing systems. Thus, this study seeks to investigate alternative ways of rearing broiler chickens for backyard/small scale broiler chicken farmers in order to increase production of animal protein (meat) via reducing the cost of building construction by adopting simple housing systems.

II. MATERIALS AND METHODS

Experimental Site: The experiment was carried out at Oluade Farms, Ilara Mokin (latitude $7^0$18N and longitude5^0 10E), Ifedore Local Government, Ondo State, Nigeria. Ilara Mokin falls within rainfall zone of the humid tropics which is characterized by hot and humid climate. The mean annual rainfall is 1500mm and the rain period is bimodal with a short break in august. The altitude is about 350.52m above sea level, the mean annual humidity is 75% and temperature is 27^0C (Oyinloye, 2013).

Pen Construction: Three (3) different rearing systems namely; deep litter housing system, colony housing system and fold unit housing system were used for this study. The deep litter system was constructed to a standard using the following materials; iron sheets, wood, wire mesh, nails, cement and blocks, while the colony cage and fold unit systems were constructed with; iron sheets, wood, wire mesh and nails and the colony cage and fold unit systems were placed under natural shades mainly plantain/banana and palm trees to provide natural cover.

Experimental Diet: One basal broiler finisher diet was formulated at the Nutrition Laboratory of the Department of Animal Production and Health, Federal University of Technology, Akure Nigeria, to meet the requirement of swine (NRC, 1994). The gross composition of the finisher diet is presented on Table 1.

Table 1: Gross Composition of Broiler Finisher Diet (g/100g)

Ingredients	
Maize	54.00
Wheat offal	5.80
Soyabean meal	21.00
Groundnut cake	12.00
Lysine	0.10
Methionine	0.10
Dicalcium phosphate	1.50
Limestone	2.00
Premix	0.25
Salt	0.25
Vegetable oil	3.00
Total	**100.00**
Calculated analysis:	
Crude protein (%)	19.70
Metabolizable energy (Kcal/kg)	3030.24
Calcium (%)	1.11
Phophorus (%)	0.50
Lysine (%)	0.10
Methionine (%)	0.40

Contained vitamins A (8,500,000 IU); D3 (1,500,000 IU); E (10,000mg); K3 (1,500mg); B1 (1,600mg); B2 (4,000mg); B6 (1,500mg); B12 (10mg); Niacin (20,000mg); Pantothenic acid (5,000mg); Folic acid (500mg); Biotin H2 (750mg); Choline chloride (175,000mg); Cobalt (200mg); Copper (3,000mg); Iodine (1,000mg); Iron (20,000mg); Manganese (40,000mg); Selenium (200mg); Zinc (30,000mg); and Antioxidant (1,250mg) per 2.5kg

Experimental Layout and Birds Arrangement: A total number of one hundred and thirty (130) started broiler chicks were procured from a vendor in Akure, Ondo State, Nigeria out of which one hundred and twenty (120) were used for this study. The started broiler chicks were divided into three (3) groups namely; deep litter, colony cage and fold unit. Each system had forty (40) started broiler chicks and each system was replicated four times (4) with ten (10) started broiler chicks per replicate in a Completely Randomized Design and the weight of each replicate was balanced (±1g). The chicks were fed *ad-libitum* on the same finisher diet throughout the experimental period (28 days), while fresh and clean water was also provided daily

throughout the experimental period. The deep litter system was covered with wood shavings as bedding materials while the colony cage and fold unit systems were not covered by any concrete or beddings but had only grasses and others plants available within the pens and environment. The colony cage system remained permanent throughout the experimental period, while fold unit system was moved on weekly basis (i.e rotational farming type) within the farm to give access to fresh vegetables and also allow the used portion to rest for some period. The Weekly feed intake and weight changes were recorded and from these two (2) parameters, feed conversion ratio was calculated.

Slaughtering of birds: At the end of the experimental period, birds were kept of feed for 12 hours so as to empty their crop to prevent carcass contamination. Two (2) birds were randomly selected per replicate for the purpose of determining the carcass characteristics. Slaughtering was done by severing the jugular vein, after stunning. The birds were bled and were scalded and defeathered. Thereafter, the dressed and eviscerated weights were expressed as a percentage of the live weight. The following organs were weighed; liver, heart, lungs, pancreas, proventriculus, spleen, gizzard and were expressed in g/kg body weight.

Blood collection: At the end of the experimental period, the birds were starved overnight and two (2) birds were randomly selected per replicate. The birds were stunned, slaughtered by severing the jugular vein, for collection of blood used for the haematological and serum indices studies. For haematology, blood samples were collected into sterilized bottles containing Ethylene Diamine Tetra-acetic Acid (EDTA) and the following blood parameters were determined; erythrocyte sedimentation rates (ESR), packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell (RBC), absolute haemoglobin and differential white blood count.

Bacteria load: Swab was inserted into the cloaca to get the feacal sample to obtain sample from the experimental birds to determine the bacteria load. The media (Nutrient Agar) used was prepared from commercially dehydrated products and reconstituted according to the manufacturer's directives, sterilized and allowed to cool. 1ml each of the serially diluted feacal sample was dropped at the centre of a Petri-dish followed by pouring of the nutrient agar using the pour plate method as described by Mumtaz *et al.* (1986). It was allowed to solidify for some minutes and then incubated at 37 °C for 24 hours. The colonies that emerged were counted and calculation for the colony forming units were expressed as log cfu/ml using the formula as described by Rukayya *et al*. (2016). The bacterial colonies that developed on the nutrient agar plates were sub-cultured by streaking on freshly prepared nutrient agar plates and MacConkey agar plates until pure colonies were obtained according to the conventional procedure as highlighted by (Fawole and Oso, 2001).

Statistical Analyses: All data collected were subjected to analysis of variance (ANOVA) using SPSS Version 17 Package and where significant differences exist, Duncan Multiple Range Test (DMRT) of the same package was used to separate the means.

III. RESULTS

Growth performance: The influence of the three housing systems on the growth performance of broiler chickens indicated that all parameters measured except the initial weight were significantly ($P<0.05$) influenced by the housing systems (Table 2). Highest final weight (2388.00±1.44g), highest weight gain (1726.75±0.32g), lowest feed intake (3475.75±36.57g) and best feed conversion ratio (2.01±0.07) were recorded in birds reared under the fold unit housing system while, lowest final weight (2305.00±2.96g), lowest weight gain (1638.75±3.66g), highest feed intake (3609.75±14.29g) and highest feed conversion ratio (2.20±0.06g) were recorded in birds under the deep litter system. In all, the weight gain and feed intake values followed a particular trend.

Table 2: Growth performance of broiler chickens reared under three different housing systems

Parameters	Deep litter	Colony cage	Fold unit
Initial weight (g)	666.25 ± 1.01	662.50 ± 0.87	661.75 ± 1.76
Final weight (g)	2305.00 ± 2.96^{b}	2307.50 ± 3.50^{b}	2388.00 ± 1.44^{a}
Weight Gain(g)	1638.75 ± 3.66^{c}	1645.00 ± 3.06^{b}	1726.75 ± 0.32^{a}
Feed Intake(g)	3609.75 ± 14.29^{a}	3480.75 ± 12.25^{b}	3475.75 ± 36.57^{b}
Feed Conversion Ratio	2.20 ± 0.06^{b}	2.11 ± 0.05^{a}	2.01 ± 0.07^{a}

*****Means without identical superscripts in the same horizontal row are significantly different ($P<0.05$)

Relative organ weights: The relative organ weights of broiler chickens reared under three different housing systems are presented in Table 3. Among all the relative organs measured, only the heart was not significantly (P>0.05) influenced by the housing systems. Highest gizzard (17.78±7.89g/kg body weight), highest lungs (5.48±0.18g/kg body weight) and highest proventriculus (4.81±0.38g/kg body weight) were observed in birds reared under the deep litter system while lowest gizzard (14.31±1.85g/kg body weight), lowest lungs (4.46±0.63g/kg body weight) and lowest proventriculus (3.41±0.23g/kg body weight) were observed in birds reared under the colony housing system. Highest pancreas (2.71±0.20g/kg body weight) and highest spleen (2.09±0.36g/kg body weight) were recorded in birds reared under the fold unit housing system while the lowest pancreas (2.40±0.09g/kg body weight) and lowest spleen (1.00±0.24g/kg body weight) were recorded in birds reared under the colony housing system. Numerically, highest heart (4.69±0.63g/kg body weight) was observed in birds reared under the deep litter system while lowest heart (3.94±0.70g/kg body weight) was recorded in birds reared under the colony housing system.

Table 3: Relative organ weight (g/kg Body Weight) of broiler chickens raised under three different housing systems.

Parameters	Deep litter	Colony cage	Fold unit
Dressed Weight (%)	92.66 ± 1.06	95.96 ± 1.00	91.45 ± 0.52
Eviscerated Weight (%)	77.81 ± 1.41	78.47 ± 1.19	77.46 ± 0.56
Liver (g/kg Body Weight)	17.39 ± 3.34^{a}	14.94 ± 0.70^{b}	17.83 ± 2.30^{a}
Heart (g/kg Body Weight)	4.69 ± 0.63	3.94 ± 0.70	4.03 ± 0.94
Gizzard (g/kg Body Weight)	17.78 ± 7.89^{a}	14.31 ± 1.85^{b}	16.55 ± 2.47^{ab}
Lungs (g/kg Body Weight)	5.48 ±0.18^{a}	4.46 ± 0.63^{b}	5.03 ± 0.80^{ab}
Pancreas (g/kg Body Weight)	2.69 ± 0.44^{ab}	2.40 ± 0.09^{b}	2.71 ± 0.20^{a}
Proventriculus (g/kg Body Weight)	4.81 ± 0.38^{a}	3.41 ± 0.23^{c}	4.05 ± 0.32^{b}
Spleen (g/kg Body Weight)	1.14 ± 0.21^{b}	1.00 ± 0.24^{b}	2.09 ± 0.36^{a}

*****Means without identical superscripts in the same horizontal row are significantly different (P<0.05)

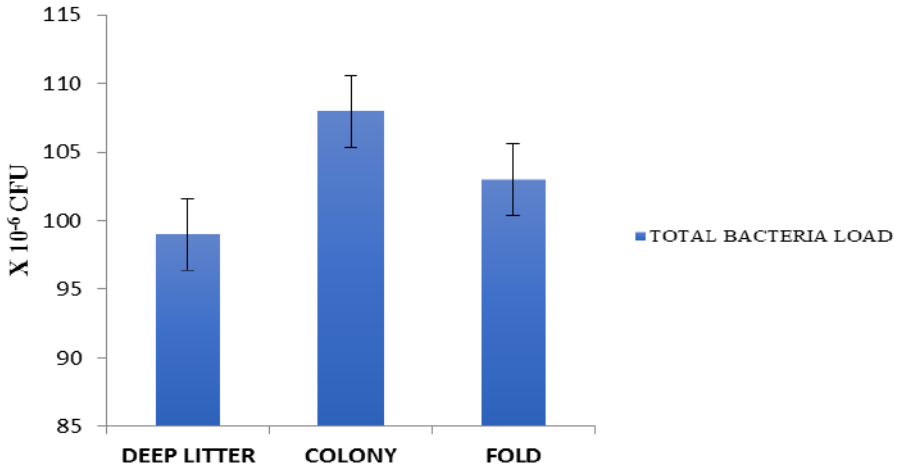
Haematological indices: The haematological indices of broiler chickens reared under the three different housing systems are presented in Table 4. Among all the parameters measured, Red blood cell, Mean corpuscular haemoglobin, Mean corpuscular volume, Lymphocytes, Heterophils and Monocytes were significantly (P<0.05) influenced by the housing systems. Highest Red blood cell (2.02±0.17×10^{6}/mm^{3}), highest Mean corpuscular haemoglobin (49.1±0.17pg of Hb), highest Mean corpuscular volume (146±0.05μ^{3}) and highest Lymphocytes (60.75±0.49%) were recorded in birds reared under colony housing system while, lowest Mean corpuscular haemoglobin (45.0±0.08pg of Hb), lowest Mean corpuscular volume (137±0.03μ^{3}) were recorded in birds reared under the conventional deep litter housing system and lowest Red blood cell (1.20±0.11×10^{6}/mm^{3}), and lowest Lymphocytes (57.00±2.35%) were recorded in birds reared under the fold unit housing system. Highest Erythrocyte sedimentation rate (2.75±0.25mm/hr) was recorded in bird reared under the conventional deep litter housing system while lowest Erythrocyte sedimentation rate (2.25±0.49mm/hr) was recorded in bird reared under the fold unit housing system. Highest Packed cell volume (28.75±1.03%) was recorded in bird reared under the colony housing system while lowest Packed cell volume (27.25±0.63%) was recorded in bird reared under the fold unit housing system. Highest Mean cell haemoglobin concentration (33.61±0.23%) was recorded in bird reared under the fold unit housing system while lowest Mean cell haemoglobin concentration (32.64±0.37%) was recorded in bird reared under the colony housing system.

Table 4: Haematological indices of broiler chickens raised under different housing system

Parameters	Deep litter	Colony cage	Fold unit
Erythrocyte sedimentation rate (mm/hr)	2.75 ± 0.25	2.50 ± 0.29	2.25 ± 0.49
Packed cell volume (%)	27.55 ± 0.49	28.75 ± 1.03	27.25 ± 0.63
Red blood cell ($x10^6/mm^3$)	1.99 ± 0.04^a	2.02 ± 0.17^a	1.20 ± 0.11^b
Haemoglobin (g/100ml)	9.25 ± 0.09	9.58 ± 0.34	9.35 ± 0.21
Mean cell haemoglobin concentration (%)	33.36 ± 0.22	32.64 ± 0.37	33.61 ± 0.23
Mean corpuscular haemoglobin (pg of Hb)	45.0 ± 0.08^b	49.1 ± 0.17^a	48.4 ± 0.15^a
Mean corpuscular volume (μ^3)	137 ± 0.03^b	146 ± 0.05^a	141 ± 0.05^{ab}
Lymphocytes (%)	58.75 ± 0.63^b	60.75 ± 0.49^a	57.00 ± 2.35^b
Heterophils (%)	25.75 ± 0.48^a	23.25 ± 0.49^b	24.50 ± 0.87^{ab}
Monocytes (%)	10.75 ± 0.48^b	11.25 ± 0.63^b	12.75 ± 0.25^a
Basophils (%)	0.88 ± 0.13	0.65 ± 0.22	0.75 ± 0.14
Eosinophil (%)	2.25 ± 0.48	2.75 ± 0.25	2.25 ± 0.25

*****Means without identical superscripts in the same horizontal row are significantly different (P<0.05)

Bacteria load: Figure 1 shows the total bacteria load of birds reared under the three housing systems. From the figure, bird reared under colony housing system had the highest bacteria load count (108×10^{-6}CFU), this is due to the fact that the cages were stationary during the experimental period, followed by bird reared under fold unit housing system with total bacterial value (102×10^{-6}CFU) and the lowest total bacterial load count (98×10^{-6}CFU) was observed in bird reared under the conventional deep litter housing system.



CFU = Coliform Form per Unit

Fig.1: Total bacteria load of started broiler chicks raised under three different housing systems

Economic analysis: The economic analysis of started broiler chicks reared under three different housing systems. From the table only the cost of started chick (₦700) was the same across the treatments. The cost of feed consumed, cost of drugs and cost of construction varied with regard to the housing system thereby leading to different values of cost of production. Highest cost of feed consumed (534.28₦/bird), highest cost of drugs (180₦/bird), highest cost of construction (340.67₦/bird) and highest total cost of production (1754.95₦/bird) were observed in birds reared under the conventional deep litter housing system while lowest cost of feed consumed (509.00₦/bird), lowest cost of drugs (130₦/bird), lowest cost of construction (136.67₦/bird) and lowest total cost

of production (1475.65₦/bird) were observed in birds reared under the fold unit housing system. Highest total sales price (2196.00₦/bird) and highest net profit (720.35₦/bird) were recorded in birds reared under the fold unit housing system while lowest total sales price (2115.00₦/bird) was observed in bird reared under the colony housing system and lowest net profit (405.05₦/bird) was recorded in bird reared under the conventional deep litter housing system (Table 5).

Table 5: Economic analysis of started broiler chick reared under three different housing systems

Parameters	Deep litter	Colony cage	Fold unit
Cost of started chicks	700.00	700.00	700.00
Cost of feed consumed (₦/bird)	534.28	515.00	509.00
Cost of drugs (₦/bird)	180.00	150.00	130.00
Cost of construction (₦/bird)	340.67	150.00	136.67
Total cost of production(₦/bird)	1754.95	1515.00	1475.65
Live weight(kg)	2.40	2.35	2.44
Selling price/kg	900.00	900.00	900.00
Total sales price(₦/bird)	2160.00	2115.00	2196.00
Net profit (₦/bird)	405.05	600.00	720.35

*As at the time of this study 25 kg bag of broiler finisher was sold at ₦3700

IV. DISCUSSION

The floorless housing systems used in this present study was designed for small-scale/backyard poultry farmers. Small groups (10 - 50 birds), controlled stocking rates and effective protection from predators make the system very suitable for small scale or backyard poultry farmers. The final weight according to production systems was in ascending order i.e from treatments deep litter – fold unit which was not in agreement with the report of Bunyamin *et al*., (2011) who reported that the average body weight of conventional bird was higher than organic control group. The results also contradicted the observations of Castellini *et al.,* (2002) who reported that outdoor organic treatments reduced growth rate when compared to conventional system. The highest feed consumed in bird reared under the deep litter housing system was compatible with findings of Bunyamin *et al.,* (2011) who reported that the highest cumulative feed intake of the deep litter system as compared with birds on outdoor systems. Also from the results of this study on feed intake, birds on colony and fold unit housing systems consumed less when compared with those in deep litter housing system. This may also be as a result of birds access to other forages within their range/pen which they also consumed and birds on deep litter housing system do not have access to such forages which makes them to concentrate mainly on given feed thereby leading to high feed intake. Also, birds on colony and fold unit housing systems consume herbs, roots, stems, leaves and invertebrates; practicing poultry with green matter can reduce the supplementation of dietary vitamins and minerals, support gut fill and can be used as enrichment device Sossidou *et al.* (2015). The result obtained in this study shows that the birds on the three housing systems have the ability to turn feed to body mass. Quality of product output for the system is fixed by a predetermined amount of kilogram carcass of live weight of broiler chickens from shed by a commercial producer (Warren and Emmert, 2000). Dressed weight and eviscerated weight of birds were not significantly different across the three housing systems and these agreed with Castellini *et al*. (2002) reports which stated dressing percentages were similar in both systems.

Haematological indices i.e Red blood cell, white blood cell, packed cell volume and haemoglobin concentration has been found useful in disease prognosis according to Togun and Oseni, (2005). The packed cell volume recorded in this study ranged between 27.23 – 28.75% and this falls within the normal range of 25 – 75% for chickens as reported by Akinmutimi (2004) and Ahamefule *et al.* (2006). Packed cell volume below normal range is an indication of anaemia. The haemoglobin concentration values recorded in this study fell within the reference values of 8.23 to 11.30 g/dl, reported for healthy broiler chickens (Olukomaiya *et al.,* 2014). There was no significant difference in the red blood cell and erythrocyte sedimentation rate of birds under the three housing systems. The heterophils which

are granulocytes of the white blood cell fell within the reference values of 10.00 to 53.60% and 0.00 to 15.00%, respectively for healthy domestic chickens (Riddell 2011). The lymphocyte is granulocytes of the white blood cell fell within the reference values of 47.2 to 85.0% (Riddell 2011) for healthy domestic chickens. The basophils obtained in this study agree with the values of 0.00 to 3.33% (Olukomaiya *et al*. 2014). The basophil values obtained suggest that there was no condition of prolonged stress. Basophils are responsible for the elaboration of histamines and heparin in circulating blood (Afolabi *et al*. 2011). The non-significant effect of different housing systems on haematological parameters of broiler chickens could be due to similar conditions of animal husbandry system used for raising the birds (Addass *et al*. 2012). The housing systems used produced no significant difference in relation to haemoglobin concentration values. This corroborates the finding of Addass *et al*. (2012) who observed same results in relation to haemoglobin concentration of the chickens that were studied. All mean values of red blood cell counts, packed cell volume and haemoglobin concentration did not differ significantly among management systems which corroborate the finding of Nyaulingo (2013).

The presence of *Staphylooccus aureus* may be due to staphylococcus from environmental factors. *Micrococcus letues* is a gram positive spore forming bacteria and heat resistant (David, 2005). Soil, humans and birds are reportedly considered as reservoirs of most of all these organisms (Fitzergerald *et al.,* 2001, Bannerman and Peacock, 2007). *Enterobacter aerogenes,* an opportunist bacterium was present in colony housing system, there are rare reports of the bacteria causing any diseases in healthy animals, *Serratia marcesces* was also present in fold unit housing system, and this also is no cause for concern as it leads to no disease in healthy animals.

The absence of *Enterobacteriaceae* family in the fold unit housing system may be associated with antibacterial activity of phytogenic plant they consumed (Bankova *et al*., 2007). It is noteworthy that few studies exist that have assessed the correlation of bacterial population in the gastro intestinal tract (GIT) with blood parameters of broiler chickens under different management systems. The present study has confirmed the heterogeneity of bacterial population (gram positive and gram negative) in the GIT of broiler chickens as documented by Gong *et al*. (2002). The diversity of bacterial distribution may probably be due to the interactions of host's tissues/cells and gut microbiota. The bacterial load did not show any significant effect on the three housing systems.

The total cost of production was highest in the conventional deep litter housing system and lowest in the fold unit housing system and this led to bird reared under the fold unit housing system recording the highest net profit over the bird reared under the conventional deep litter housing system and this simply means that backyard farmers using the fold unit housing systems will have more cash than farmers using the conventional housing system.

V. CONCLUSION

Based on the findings, an inference could, thus, be drawn from the present study that broilers reared on floorless pastured pen exhibited better growth performance with optimum feed intake leading to best feed conversion ratio as compared to those reared under deep litter housing system. The summarized results presented here support the view that there is a promising potential for backyard farmers especially those rearing broiler chickens for Christmas and New Year festival periods. Weekly movement of the fold unit pens do not support coccidiosis outbreak.

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Bacteriological Quality of Citrus Fruits (Morocco)

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Abstract— *In our research, we studied the bacteriological quality of citrus fruits. Thus, the bacterial microflora associated with citrus fruit is quite varied and diverse. It is usually a saprophyte flora easily be destroyed by moist heat. This plant belongs to the family Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, Micrococcaceae and Bacilaceae. This contamination is often untreated manure, organic fertilizer from a bad composting, irrigation water not clean. The hands of workers who come into contact with fruit are also a potential source of contamination across the production system of the juice, ie at all these stages: growing, harvesting, processing, packaging and transportation. This contamination is usually faecal origin toilet (hands of workers contaminated by fecal matter).*

The presence of E. Coli and Streptococcus feacalis is evidence of such contamination. Indeed, the presence of these bacteria indicates that other pathogens present in the feces of sick individuals or healthy carriers may be present (Salmonella, Vibrio, Clostridia...) what constitutes a risk of contamination of the entire production. Among the most dangerous pathogens that can be seen quite often on the fruit, quoting E. coli O157: H7, Salmonella spp.... Colereae Vibrio, which are Gram negative, the wall, composed of lipopolysaccharide (LPS), is an endotoxin. The ingestion of endotoxin causes a water leak at the level of the enterocytes (cells of the intestine) and consequently dehydration of the individual addict.

Keywords— ***citrus fruits, contamination, microbiological quality, bacterial microflora..***

Qualité bactériologique des fruits d'agrumes (Maroc)

Résumé— *Dans notre travail de recherche, nous avons étudie la qualité bactériologique des fruits d'agrumes. Ainsi, La microflore bactérienne associée aux fruits d'agrume est assez variée et diversifiée. Il s'agit généralement d'une flore saprophyte facilement destructible par la chaleur humide.*

Cette flore appartient aux familles des Enterobactériaceae, Vibrionaceae, Pseudomonadaceae, Bacilaceae et Micrococcaceae. Cette contamination provient souvent du fumier non traité, d'engrais organique provenant d'un mauvais compostage, d'eau d'irrigation non propre. Les mains des ouvriers qui entrent en contact avec les fruits représentent aussi une source potentielle de contamination à la grandeur du système de production du jus, c'est à dire à toute ces étapes : culture, récolte, transformation, emballage et transport.

Cette contamination est généralement fécale originaire des toilettes (mains des ouvriers souillées par la matière fécale). La présence d'E.coli et de Streptococcus feacalis constitue une preuve de cette contamination. En effet, la présence de ces germes signifie que d'autres germes pathogènes présents dans la matière fécale des individus malades ou porteurs sains peuvent être présents (salmonelles, vibrions, clostridies….) ce qui constitue un risque de contamination de toute la production.

Parmi les germes pathogènes les plus redoutables qu'on peut rencontrer assez souvent sur les fruits, on cite E.coli O157 : H7 , Salmonella spp., Vibrio colereae…, qui sont des bactéries Gram négatif dont la paroi, formée des lipopolysaccharides (LPS), constitue une endotoxine. L'ingestion de ces endotoxines provoque une

fuite d'eau au niveau des enterocytes (cellules de l'intestin) et par voie de conséquence une déshydratation de l'individu intoxiqué.

Mots clés— Fruits d'agrumes, contamination, qualité microbiologique, microflore bactérienne.

I. INTRODUCTION

Les infections des fruits d'agrumes par l'agent pathogène peuvent se produire aussi bien avant qu'après la récolte. Les infections des fruits d'agrumes sont initiées par des contaminations au champ, quelques jours à plusieurs semaines avant la récolte. Ces infections sont favorisées, entre autres, par un taux d'inoculation élevé dans l'air et par une grande humidité. La détérioration peut être limitée par des conditions défavorables de l'environnement et également par une résistance de l'écorce du fruit (Douyle et Padhye, 1989 ; Doyle et Cliver, 1990 ; Beuchat et Ryu, 1997 ; Jackson, 2001 ; Forrest, 2001).

Les portes d'entrées des agents pathogènes en cours et après la récolte (stockage) des agrumes sont les blessures et les microlésions accidentelles (Faber, 1989. Tabaght, 1994).

Dans cette partie de notre travail, nous allons examiner la qualité des fruits d'orange tout en exposant les principaux paramètres physico-chimiques, nutritionnels et techniques susceptibles d'influer sur la qualité. Nous allons mettre en relief l'importance de certaines espèces de bactéries dans la dégradation de la qualité des fruits d'orange.

II. MATERIEL ET METHODES

1/ Matériel biologique

a/ Fruits des agrumes

Les fruits, sujets de notre étude proviennent des plantes fruitières appartenant aux espèces suivantes :

* Clémentinier (*Citrus clementina* Hort. ex. Tan.),

* Mandarinier (*Citrus reticulata* Blanco),

* Oranger (*Citrus sinensis* Osb),

* Citronnier (*Citrus limon* L.),

* Pomelo (*Citrus paradisi* Macf.).

2/ Echantillonnage

* **Fruits d'agrumes** : pour le contrôle des agrumes nous avons choisi les niveaux suivants:

- station réception d'agrumes

- station brossage-lavage à l'avant et après sortie.

On prend 1 kg de fruit comme un échantillon représentatif. Les fruits sont lavés par l'eau distillée stérile à raison de 250 ml pour 1 kg d'échantillon.

L'analyse de l'eau de lavage nous donne des indications sur le degré de contamination des fruits par des souches pathogènes (bactéries ou champignons).

3/ Culture et identification des microorganismes (bactéries et champignons)

3-1/ Milieux de culture

3-1-1/ Milieu Orange Serum Agar (OSA)

C'est un milieu de culture qui permet le développement de toute la flore qui exige des facteurs de croissance présents dans l'extrait d'orange.

Les bactéries, champignons, (levures et moisissures) adaptés aux jus d'orange se cultivent sur ce milieu.

Le milieu OSA contient en g/l (Tryptone, 10 ; extrait de levure, 3 ; extrait Orange, 5 ; Glucose, 4 ; Phosphate dipotassique, 3 ; Agar bactériologique, 17)

Le milieu est stérilisé à l'autoclave à 120 °C / 15 min, puis coulé en boîtes de Petri stérilisées à l'autoclave (120°C/30 min) ou à sec au four (170°C/1h).

3-1-2/ Gélose nutritive

La gélose nutritive est un milieu qui convient à la culture des germes ne présentant pas d'exigences particulières. Ce milieu est composé de (en g/l): (Peptone, 5 ; Extrait de viande, 1 ; Extrait de levure, 2 ; Chlorure de sodium, 5 ; Agar, 15)

Ce milieu est stérilisé à l'autoclave (120°C/15min) et coulé en boîte de Petri ou en tube incliné pour la conservation des souches pures. Dans ce cas, la conservation se fait à 4°C/ 1mois.

3-1-3/ Milieu à base de pomme de terre (PSA)

Des petits cubes de 200 g de pommes de terre non pelées et vieilles de préférences, sont lavés, coupés et placés dans un litre d'eau portée à ébullition pendant 1 heure. Après ébullition, les pommes de terre sont écrasées puis filtrées et le volume est complété à 1 litre.

Ce mélange est ensuite additionné de 15 g de saccharose et 20 g d'agar. Le pH du milieu est ajusté à 5,6 et stérilisé à l'autoclave à 120 °C/15 min.

Le milieu PSA est utilisé pour l'isolement, la culture et le dénombrement des champignons.

Ce milieu est particulièrement recommandé pour la mise en évidence des contaminations dans les produits alimentaires (ex: jus d'orange).

3-1-4/ Bouillon nutritif

Ce milieu a la même composition que la gélose nutritive mais sans agar bactériologique.

Ce milieu, coulé en tubes et stérilisé à l'autoclave, sert généralement aux préparations de précultures et aux enrichissements.

3-1-5/ Milieu TCBS

C'est un milieu sélectif qui sert à isoler les germes de vibrions après enrichissement sur milieu riche contenant 40 g de peptone et 60 g de Nacl par litre.

Le milieu TCBS a la composition suivante (en g/l) : (Peptone, 10 ; Extrait de levure, 5 ; Citrate de sodium, 10 ; Thiosulfate de sodium, 10 ; Chlorure de sodium, 10 ; Bile de bœuf, 8 ; Citrate ferrique, 1 ; Saccharose, 20 ; Bleu de thymol, 0,04 ; Bleu de bromothymol, 0,04 ; Agar ; 14). Ce milieu est chauffé jusqu'à ébullition mais non autoclavé.

Après ensemencement, les boîtes sont incubées 15 à 18 heures à 35°C, période suffisante pour l'apparition des colonies des vibrions alors que la totalité des autres germes est inhibée durant les premières heures d'incubation. Les colonies de vibrions sont caractéristiques de quelques espèces:

- *V. parahaemolyticus* : petite colonie à centre vert ou bleu à bord incolore

- *V. cholerea* : petite colonie jaune

- *V. aglinolyticus* : grosse colonie jaune-brun

Les colonies suspects sont repiquées sur milieu Kligler salé à 3% pour identification (voir milieux d'identification).

3-1-6/ Milieu King A et King B.

Ces milieux servent à l'isolement des *P*seudomonas.

Les milieux King A (ou P) et King B (ou F) permettent de différencier les espèces pigmentées de *Pseudomonas*.

Ces milieux ont la composition suivante (en g/l): pour milieu King A (ou P) on a : (Peptone de gélatine, 20 ; Glycérol , 10 ; Sulfate de potassium, 10 ; Chlorure de magnésium anhydre, 1,4 ; Agar, 15). La stérilisation se fait à l'autoclave à 120°C/15 mn.

Pour le milieu de King B (ou F) on a : (Protéose peptone n° 3 ou polypeptone (BBL), 20 ; Glycérol, 10 ; Phosphate, 1,5 ; Sulfate de magnésium ($7H_2O$), 1,5 ; Agar, 15). Ce milieu est stérilisé à l'autoclave à 120°C/15 min.

Le milieu A favorise la production de pyocyanique du bacille pyocyanique ou *Ps. aerogenosa* alors que le milieu B favorise la fluorescine (Pyoverdine) de *Ps. fluorescens*, *Ps.putida*, et divers autres *Pseudomonas*.

Une boîte de milieu A et une boîte de milieu B sont ensemencées avec une anse de culture prise dans un bouillon nutritif. Après incubation à 30°C/ 4 jours, les colonies suspectes sont repiquées sur milieu Kligler pour la suite d'identification.

3-2/ Ensemencement

3-2-1/ Ensemencement pour isoler des souches

Pour isoler une souche, en pratique un ensemencement par épuisement sur un milieu de culture approprié à partir des fruits, de jus ou autres.

Après incubation à 30°C/48h, les colonies isolées sont ensemencées à nouveau comme précédemment pour s'assurer de la pureté de la souche.

Les souches diffèrent normalement par leurs aspects culturaux (forme de la colonie, aspect, couleur, élévation, consistance,…). Chaque type de colonie est repiqué séparément et conservé sur gélose nutritive inclinée (pour les bactéries) ou sur PSA incliné (pour les levures et les moisissures) en vue d'une identification complète.

3-2-2/ Dénombrement de la microflore du jus et des fruits.

Une série de dilution du jus semi-fini ou de l'eau de lavage est réalisée pour obtenir une suspension bactérienne dénombrable sur milieu solide en boîte de Petri. Des dilutions de 10 en 10 sont réalisées et l'ensemencement se fait par étalement de 0,1 ml de chaque dilution sur le milieu de culture OSA dans des conditions aseptiques.

Les boîtes sont ensuite incubées inversées dans l'étuve à 30 °C/48h.

L'ensemencement peut être aussi réalisé par la méthode d'ensemencement en masse, mais cette méthode sous estime la taille de la population par le fait que les souches très exigeantes en oxygène ne se développent pas.

Chaque souche vivante introduite dans la masse d'un milieu gélosé favorable donne en principe naissance à une colonie repérable à l'œil nu. En conséquence, si un produit ou sa dilution est ensemencé dans ce milieu de culture, le nombre de colonies développées correspond au nombre de micro-organismes présent dans le volume inoculé.

3-3/ Identification

3-3-1// Etude macro et microscopique

a/ Observation des colonies

Après incubation, tous les caractères macroscopiques des colonies sont notés.

- Forme de la colonie : circulaire, irrégulière ou rhizoïde.

- Aspect : punctiforme (<1 mm de diamètre), moyenne, grande ou envahissante.

- Opacité : transparence, translucidité.

- Elévation : colonie plate, convexe, centrée, surélevée.

- Surface : lisse, rugueuse, émoussée, brillante, sèche, poudreuse, plissée, crémeuse.

- Bord : entier, ondulé, lobé, denté, rhizoïde, crénelé.

- Consistance : visqueuse ou granulaire.

- Odeur : présence ou absence d'odeur caractéristique.

Chaque type de colonie est repiqué stérilement dans un tube de bouillon nutritif stérile, après purification.

b/ Observation microscopique.

*** A l'état frais:** chaque souche (bactéries) est observée au microscope entre lame et lamelle avec ou sans colorants (bleu de méthylène). On note généralement la forme, la taille, l'arrangement et pour les champignons on note en plus la structure.

*** Après coloration de Gram pour les bactéries:** La coloration de Gram permet de séparer deux grands groupes de bactéries: Gram + et Gram -. Les premiers ont une paroi épaisse et sans LPS, ces types de bactéries se colorent en violet car l'alcool ne pénètre pas dans la cellule alors que les seconds ayant une paroi mince et riche en LPS se colorent en rose parce que l'alcool, au cours de la coloration, détruit le complexe cristal violet-lugol: les cellules décolorées prennent la couleur rose de la fushine.

L'observation microscopique, après coloration de Gram, donne la forme, la taille et le type de Gram et même la présence ou absence de spores chez certaines souches notamment les lactobacilles.

3-3-2/ Etude des caractères biochimiques des bactéries

Après avoir isolé une bactérie, il faut procéder à son identification. Une première classification est faite d'après les caractères morphologiques et culturaux puis l'identification des espèces et menée grâce à des milieux d'identification. Certains milieux d'identification sont spécifiques de certains germes, d'autres peuvent servir à de nombreux germes.

La première condition à respecter est d'avoir un germe à l'état "pur", et une culture abondante et suffisante pour ensemencer différents milieux d'identification.

3-3-2-1/ Milieu Kligler-Hajna

Ce milieu coulé en tube de manière à former un culot et une tranche permet de donner 4 caractères: fermentation du glucose, du lactose, production de gaz et de H_2S.

Ce milieu à la composition suivante (en g/l) : (Peptone, 20 ; Nacl, 5 ; Glucose, 1 ; Lactose 10 ; Thiosulfate de sodium, 0,3 ; Citrate ferrique, 0,3 ; Extrait de viande de bœuf, 3 ; Extrait de levure, 3 ; Rouge de phénol, 0,05 ; Agar, 12).

L'ensemencement se fait au fil droit: une strie sur la tranche et piqûre centrale en profondeur dans le culot. Après 24 heures d'incubation à 37°C, on obtient les informations suivantes:

* Si la souche est lac (-) et glu (+): il y a d'abord acidification du milieu dans le culot car il y a utilisation du glucose par fermentation, mais cette fermentation est rapidement neutralisé par consommation des peptones dans la pente. En effet, contrairement à la tranche, au niveau du culot il y a une grande quantité de glucose par rapport au nombres de cellules ensemencées, donc durant 24 h c'est surtout le glucose qui est attaqué.

* Si la souche est lac(+) et glu (+): Il y a acidification par fermentation du glucose prolongée par la suite par celle du lactose au niveau de la tranche. L'acidification se traduit par une coloration jaune du milieu (virage de l'indicateur coloré: rouge de phénol).

* La production de H_2S se traduit par noircissement du milieu qui débute à la jonction pente-culot due à la production de sulfate de fer.

* La fermentation des sucres se traduit parfois par production de CO_2. On aura soit formation de pochette de gaz dans la gélose ou parfois même soulèvement de la gélose au niveau du culot.

Dans le cas d'identification des vibrion, nous avons utilisé le même milieu de Kligler mais à 3% de Nacl car les vibrions exigent un milieu de culture salé.

3-3-2-2/ Recherche de l'indole

Le milieu utilisé est une eau peptonée constituée de peptone pancréatique ou peptone dite bactériologique exempte d'indole.

Le milieu a la composition suivante (en g/l): (peptone exempte d'indole, 10 ; Chlorure de sodium, 5). Le milieu est stérilisé par autoclavage à 120°C/15min est ensemencé et incubé à 37°C pendant 24 h. La révélation se fait après l'addition au milieu de 1 ml de réactif de Kovacs dont la composition est la suivante: (Paradimethylamino-benzaldéhyde, 5 g ; Alcool amylique,75 ml ; HCl concentré, 25 ml).

La présence d'indole se manifeste par l'apparition d'une coloration rouge cerise.

La formation d'indole à partir du tryptophane est un caractère propre à certaines espèces bactériennes, donc utile pour leur identification.

3-3-2-3/ Production de l'uréase

a/ sur milieu urée de Christensen

Le milieu à l'urée de Christensen est un milieu solide qui sert à l'identification de certains germes, par la mise en évidence de l'hydrolyse de l'urée.

Formule (en g/l d'eau distillée) : (Peptone, 1 ; Chlorure de sodium, 5 ; Glucose, 1 ; Phosphate monopotassique, 2 ; Urée, 24 ; Rouge de phénol, 0,012 ; Agar, 17,5). Ce milieu est stérilisé à l'autoclave à 120°C/15min et coulé en tubes sous forme de tranche.

La tranche est ensemencée en stries serrées et parallèles avec une culture provenant d'un milieu solide. L'incubation se fait à 37°c pendant 1 à 6 jours tout en évitant la dessiccation du milieu.

L'hydrolyse de l'urée se traduit par une alcalinisation du milieu qui prend une teinte rosée, puis rouge, d'abord sur la tranche puis dans le culot (ex. *Proteus*).

Sur milieu de Stuart (test rapide):

C'est un milieu liquide, jaune orangé, synthétique, de pH 6,8 contenant de l'urée, des phosphates disodiques et monopotassiques, l'extrait de levure et le rouge de phénol. Le milieu est stérilisé par filtration.

On incube à 37°C une suspension de germes dans 0,5 à 1 ml de milieu Stuart.

Si le germe est capable d'utiliser l'azote de l'urée pour se multiplier, l'uréase élaborée dégradera l'urée selon la réaction suivante:

$$CO(NH_4)_2 \longrightarrow CO_2 + 2NH_3 + H_2O$$

Il en résulte une alcalinisation et le milieu deviendra rouge violacé en 3 à 5 heures.

3-3-2-4/ Recherche de la β-galactosidase (Test O.N.P.G)

Cette réaction consiste à rechercher directement dans la cellule bactérienne l'existence d'une enzyme permettant l'utilisation du lactose.

Chez les bactéries fermentatives, l'hydrolyse du lactose en glucose et galactose est réalisée par une enzyme intracellulaire : la β-galactosidase.

Certaines entérobactéries sont dites lactose lent, parce qu'elles possèdent le gène béta-galactose et ne possèdent pas le gène perméase.

En fait les lactoses lents possèdent la possibilité de fermenter le lactose ce qui permet de les rapprocher des germes lactose (+)

La mise en évidence de la β-galactosidase est donc un caractère primordial.

A partir de Kligler, on réalise une suspension dense des bactéries dans 1 ml d'eau distillée. On ajoute ensuite un disque d'ONPG. Si les bactéries possèdent une β-galactosidase, la solution devient jaune, après un séjour de 20 min à 4h /37°C.

3-3-2-5/ Réaction au rouge de méthyle (RM) et de Vosges-proskauer (V.P)

On utilise un milieu de culture Clark et Lubs (en g/l): Peptone, 5; Phosphate bipotassique, 5; Glucose, 5) qu'on inocule par une culture jeune de 18 h. Après incubation de 48 h à 37°C, on deux tubes:

* un tube sert à vérifier la production d'acides par addition de Rouge de méthyle (RM) à 0,2% dans l'alcool à 60°. Si le milieu prend :

- une teinte rouge (pH inférieur à 4,2): réaction positive (RM+)

- une teinte jaune (pH supérieur à 6,3): réaction négative (RM-).

La réaction de RM est liée au pH du milieu. En effet, de nombreuses bactéries produisent des composés organiques acides à partir de l'acide pyruvique.

***** dans un deuxième tube on décèle la production d'Acétyl Méthyl-Carbinol ou Acétoïne: Réaction de Vosges-Proskauer. Ce composé est un produit de dégradation de l'acide pyruvique.

Après 48h d'incubation en Clark et Lubs, 1 ml de milieu et additionné de 0,5 ml d'alpha Naphtol à 6% dans l'alcool absolu et de 0,5 ml d'une solution aqueuse de NaOH à 16%. On agite et on attend 15 minutes.

Si la souche étudiée produit de l'acéthylméthyl-carbinol, la présence de ce métabolite se manifeste par l'apparition d'une coloration rouge en surface pouvant diffuser dans le milieu (souche VP+).

3-3-2-6/ Gélose à la phénylalanine désaminase ou gélose APP

Ce milieu très utile pour la classification des Entérobactéries lac-, permet de rechercher la transformation de la phénylalanine en acide phénylpyruvique.

Ce milieu est composé de (en g/l): Peptone, 10 ; Phosphate bipotassique, 1 ; Chlorure de sodium, 5 ; Extrait de levure, 3 ; D.L phénylalanine, 2 ; Agar, 12). La stérilisation se fait

à l'autoclave à 120°C pendant 20 minutes puis le milieu est refroidi en position inclinée pour donner une tranche.

Après incubation de 18 à 24 heures à 37 °C, la culture est couverte:

+ Soit avec 5 ou 6 gouttes du réactif suivant : (Solution de demi-saturation d'alun de fer, 5 ml ; Sulfate d'ammonium, 2 g ; Acide sulfurique à 10%, 1 ml).

+ Soit avec 5 ou 6 gouttes d'une solution au 1/3 de perchlorure de fer.

L'apparition rapide d'une coloration verte franche est caractéristique de la transformation de la phénylalanine en acide phényl-pyruvique.

3-3-2-7/ Milieu citrate de Simmons.

Ce milieu synthétique permet de savoir si la souche est capable d'utiliser le citrate comme seule source de carbone. Il est composé (en g/l) de: (Sulfate de magnésium, 0,2 ; Citrate de sodium, 2 ; Chlorure de sodium, 5 ; Phosphate d'ammonium, 0,2 ; Phosphate d'ammonium monosodique, 0,8 ; Bleu de bromothymol, 0,08 ; Agar, 15). La stérilisation se fait à 121°C pendant 15 minutes suivie de refroidissement en position inclinée pour obtenir une pente.

Ce milieu est ensemencé à partir d'une culture prélevée sur milieu gélosé. L'ensemencement se fait en surface par une strie centrale est longitudinale. Après Incubation à l'étuve à 37°C pendant 48 h, on observe la couleur et la culture:

* Citrate (+): culture rapide, alcalinisation : milieu bleu

* Citrate (-) : milieu reste vert et sans culture.

3-3-2-8/ Mobilité de la souche

Nous avons utilisé un milieu de culture dit ″gélose mobilité ″. Ce milieu a la composition suivante (en g/l) : (peptone,10 ; NaCl, 5 ; Agar, 5). C'est un milieu mou (semi solide) est coulé en tubes et ensemencé par piqûre centrale avec fil droit.

Après incubation de 24 à 48 h /37°C, lorsque les germes sont mobiles, la culture diffuse de part et d'autre de la piqûre et le milieu devient trouble. Si le germe est peu mobile, seulement quelques bourgeons se développent de part et d'autre de la ligne centrale. S'il est immobile, il cultive uniquement sur le trait de la piqûre.

3-3-2-9/ Mise en évidence des enzymes respiratoires

a/ Oxydase

On prélève le germe à partir d'un milieu solide avec öse et on le dépose sur du papier imprégné d'oxalate de dimethyl-para-phénylène-diamine, en solution à 1% dans l'eau distillée stérile.

Le teste se déroule à l'abri de la lumière. Le papier imprégné doit être incolore .

Si le germe possède une oxydase, une coloration de la strie en violet survient immédiatement.

Ce test est d'une importance capitale puisque toute la famille des *Entérobactériaceae* est OX^-.

b/ Catalase

Ce test est pratiqué pour les bactéries bacille ou cocci à Gram +.

Le germe est prélevé à partir d'un milieu de culture solide et mis en suspension dans l'eau oxygénée à 10%. La présence de la catalase se traduit immédiatement par dégagement d'oxygène :

$$H_2O_2 \longrightarrow H_2O + ½\ O_2$$

III. RESULTATS

La flore présente dans le jus de différentes stations n'est autre que la résultante d'une variété de micro-organismes issus du fruit lui même, de l'atmosphère de stockage, de la chaîne de transformation et de conditionnement, de l'eau et du personnel. La qualité microbiologique du produit destiné à la consommation est une composante capitale pour garantir une sécurité hygiénique.

6/ Principales bactéries associées aux fruits.

Les souches fréquemment isolées de différentes étapes de la production de jus d'orange se répartissent en 2 groupes : bacilles à Gram + mais le plus souvent des bacilles à Gram-. Dans ce dernier cas, les bactéries appartiennent à 2 familles les *Pseudomonadaceaes* et les *Entèrobactériaceaes* (tableau 2).

Tableau 1: Origine des souches identifiées

souches	Origine
Pseudomonas sp El 1	Eau de lavage
Pseudomonas sp El 2	Eau de lavage
Pseudomonas sp El 3	Eau de lavage
Klebsiella El 4	Eau de lavage
Pseudomonas sp El 5	Eau de lavage
Enterobacter El 6	Eau de lavage
E. coli El 7	Eau de lavage
Proteus El 8	Eau de lavage
Proteus El 9	Eau de lavage
Serratia El 10	Eau de lavage
Levinia El 11	Eau de lavage

Citrobacter El 12	Eau de lavage
Baccillus El 13	Eau de lavage

***EL** : Eau de lavage*

Si les eaux de lavage des fruits renferment une flore très diversifiée et marquées par la présence des *Pseudomonas* et *Klebsiella* provenant probablement des eaux d'irrigation ou plutôt des fumiers non traités, au contraire les produits finis ne renferment, après pasteurisation, que les bactéries banales de la familles des *Entèrobactèriacaes*, sauf un cas où on a trouvé un *bacille* à endospore cultivant dans une atmosphère normale et dont l'origine peut être attribuée à une contamination par l'air et un traitement inefficace des conduits de la production du jus.

Les souches isolées ont les propriétés biochimiques et physiologiques groupées dans le tableau 2.

Tableau 2: Caractères biochimiques et physiologiques des souches

test	**Souches**												
	El 1	El 2	El 3	El 4	El 5	El 6	El 7	El 8	El 9	El 10	El 11	El 12	El 13
Forme	B	B	B	B	B	B	B	B	B	B	B	B	B
Gram	G^-	G^-	G^-	G^-	G^-	G^-	G^-	G^-	G^-	G^-	G^-	G^-	G^+
Ox	+	+	+	+	-	-	-	-	-	-	-	-	
Mobilité	++	+	+/-	+	++	+	+	+	-	++	+	-	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	
Lactose	+	+	-	-	+	+	-	-	+	-	+	+	
H_2S	-	-	-	-	-	-	+	+	-	-	+	+	
Gaz	+	+	-	-	+	+	-	+	+	-	-	+	
Citrate					+	-	-	-		+/-	+	+/-	
Indole					-	+	-	-		-	+	-	
RM					-	+				+	+		
VP					+	-				-	-	-	
Malonate de Na							-	-		-		-	
Uréase					-	-	+	+		-	-	-	
ONPG					+	+	-	-		-	-	-	
King A	+	-											
King B	-	+											
Spore													+

IV. DISCUSSION ET CONCLUSION

Le jus d'orange est riche en eau et en sucres facilement métabolisables par les microorganismes ; c'est un milieu de culture par excellence (Forrest, 2001).

Ces conditions exposent facilement ces produits à la contamination par les champignons et les bactéries pathogènes, une contamination simple ou multiple du Jus peut provenir de nombreuses sources: le sol, l'eau, les engrais (surtout le compost), les travailleurs, le matériel agricole et les conditions de stockage et de transformation.

La flore présente dans le jus n'est autre que le résultante d'une variété de micro-organismes issus du fruit lui même, de l'atmosphère de stockage, parfois de la chaîne de transformation et de conditionnement, de l'eau, de l'air ou même du personnel (Attrassi et Badoc, 2014).

Malgré l'effort entrepris pour réduire la charge des microorganismes dans l'air de l'industries ou pour atténuer la flore liée aux fruits, les quelques cellules de bactéries ou de champignons qui peuvent échapper au contrôle trouvent dans le jus un milieu tout à favorable à leur croissance et à leur reproduction. En effet, le contrôle microbiologique de

produits finis vise à tuer les microorganismes et inactiver les enzymes responsables de l'altération du produit, mais quelques germes peuvent persister. Ces germes sont en effet considérés loin d'être pathogènes mais sont considérés comme germes nocifs, capables d'altérer le produit à cause de leur métabolisme (D'oust, 1989, 1997).

Les bactéries que nous avons isolées dégradent tout le glucose et se caractérisent par une croissance rapide. Les champignons isolés tolèrent plus l'acidité du milieu (jus d'orange) et peuvent se multiplier sur des substrats plus complexes.

En effet, Salmaoui (1999) avait déjà montré que sur les différentes sources de carbone, les sucres monosaccharides (glucose, fructose et galactose) et le disaccharide (saccharose) sont les plus assimilés par les différents champignons testés (Chapot et Dleuchi, 1964). Le glucose est biologiquement la meilleure source de carbone pour la croissance mycélienne des champignons, ce qui concorde avec le concept général des champignons ″tous les champignons possèdent la capacité de convertir le fructose en un dérivé phosphoryle du glucose capable de s'intégrer dans la principale voie de la chaîne respiratoire des enzymes extra cellulaire chez les souches capables de dégrader les polysaccharides″ (Attrassi et Rahouti, 2016).

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The Growth Responses of Potato Crops (*Solanum tuberosum* L.) in various type of Rhizobacteria and Mycorrhiza

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Abstract— *Potato (Solanum tuberosum L.) is a plant prefetch queue of five seasonal vegetables that are much in demand in Indonesia. Potato needs tend to increase every year in line with population growth and development of the food processing industry are made from raw potatoes. In general, potato production in Indonesia is still relatively low and still do to meet their import requirements. But the government has always sought to boost the national potato production. One of the efforts to improve potato production with the use of Plant Growth Promoting Rhizobacteria (PGPR) and fungi Mycorrhizal Fungi (AMF). The use of microorganisms PGPR and Mycorrhizae naturally associated with plant roots and have the ability to improve plant growth. The purpose of this study is to obtain the best interaction between species rhizobacteria and mycorrhiza on the growth of potato plants. This study is a two-factor factorial experiment with three replications in a randomized block design. First factor is type rhizobacteria which are without rhizobacteria, RZ3.L2.1, RZ3.L2.2, RZ3.L2.5. The second factor is the type of mycorrhizal wich are Glamus mycorrhiza, mycorrhizal and mycorrhizal Acaulospora sclerocystis, Data were analyzed by analysis of variance and DNMRT 5%. The results showed that the best treatment for the growth of the potato crop is rhizobakteria RZ3.L2.2 with mycorrhizal glamus.*

Keywords— ***potatoes, rhizobacteria, arbuscular mycorrhizal fungi.***

I. INTRODUCTION

Potato (Solanum tuberosum L.) is one of the vegetables that are in Indonesia. Potatoes contain carbohydrates and high nutrition. In Indonesia, potatoes can also be used as an alternative food in addition to rice(1). Chips also includes plants prefetch queue of five seasonal vegetables. Potatoes are widely cultivated in Indonesia consists of three types of color bulbs. Yellow bulbous potato-like Granola, Cipanas, Cosima, and Thung 151 C. bulbous white potatoes such as Diamant and Marita. Potatoes red bulbous like Desiree and Kondor.

Potato needs tend to increase every year in line with population growth and development of the food processing industry are made from raw potatoes. But the market demand can not be met in terms of both quantity and quality. In general, potato production in Indonesia is still relatively low at 1,164,738 tons and Indonesia still imports of potatoes amounted to 10,452 tons worth US $ 4.65 million(2). Low national potato production to meet demand is influenced by several things including which agricultural land is increasingly narrowed from year to year, pests and diseases and the use of pesticides and inorganic fertilizers were excessive.

One effort to address the problem is by the use of Plant Growth Promoting Rhizobacteria (PGPR) and fungi Mycorrhizal Fungi (AMF). The use of microorganisms naturally PGPR in association with plant roots and have the ability to improve plant growth is a biological control techniques that lately developed rapidly. The use of rhizobacteria as biological agents that stimulate plant growth and increase crop yields predicted it would be an interesting study that continues to grow in agriculture in the future(3).

II. MATERIALS AND METHODS

Penelitian di Nagari Baruah Gunuang Kecamatan Bukit Research in Nagari District of Bukit Barisan Gunuang Baruah, District Fifty Cities with altitude ± 1000 masl. The experiment was conducted from November 2019 -Februari in 2020 and preparation of bacterial isolates Mikrobiology indigenous conducted at the Laboratory, Faculty of Agriculture, University of Andalas Padang, Materials used are seed potatoes, isolates Rhizobakteri, FMA, water, paper labels, plastic samples, envelopes, manure, lime, fertilizers NPK, The experiment was arranged according to the design of 4 x 3 with two factors in a randomized block design (RBD) with three replications. The treatments were

kind Rizobakteri (R) and the type of Mycorrhizal Fungi Fungi (M). Type Rizobakteri (R) provided consisted of four types:
(R0) Without Rhizobakteri
(R1) RZ3.L2.1
(R2) RZ3.L2.2
(R3) RZ3.L2.5
Dose Mycorrhiza Fungi Fungi (M) Multispora namely:
(MG) mycorrhizal glamus
(MA) mycorrhizal Acaulospora
(MS) mycorrhizal sclerocystis
Data were analyzed by analysis of variance, if the F-treatment count is greater than F-table then continued with Duncan's New Multiple Range Test at 5% level.

III. RESULT AND DISCUSSION

1. Ability Test Gas Hydrogen Cyanide (HCN)

Table 1 shows that there are eight treatment has the ability to produce a high HCN and 4 treatment resulted in a low HCN.

Table 1. Effect of Mycorrhizal Fungi Fungi Rhizobakteri and against HCN Potato

Rhizobakteria	mycorrhizal	Discoloration	HCN
Tanpa Rhizobakteri	Mikoriza glamus	Orange	+++++
	Mikoriza acaulospora	Orange	+++++
	Mikoriza sclerocystis	Orange	++++
RZ3. L2.1	Mikoriza glamus	Brown	+
	Mikoriza acaulospora	Brown	+
	Mikoriza sclerocystis	Orange	++++
RZ3. L2.2	Mikoriza glamus	Orange	+++
	Mikoriza acaulospora	Orange	+++
	Mikoriza sclerocystis	Orange	+++
RZ3. L2.5	Mikoriza glamus	Brown	+
	Mikoriza acaulospora	Brown	+
	Mikoriza sclerocystis	Orange	+++

++++ and +++ = high HCN, + = low HCN

Table 1 also shows that the treatment has the ability to produce a high HCN will be orange and brown lower. The color change caused by bacteria of sodium cyanide (NaCN), which is a combined reaction of picric acid / Na2CO3 with cyanide. NaCN formed through cyanide gas absorption by NaOH or Na2CO3 through the reaction between sodium and ammonia that initially will be formed NaNH2 that will react with the carbon and will produce sodium cyanamide (Na2NCN) and eventually will be formed NaCN which is one type of cyanide. This shows that the brighter the color, the higher the isolates caused more HCN content of sodium cyanide were joined by ammonia (4). The color differences can be seen in Figure 1.

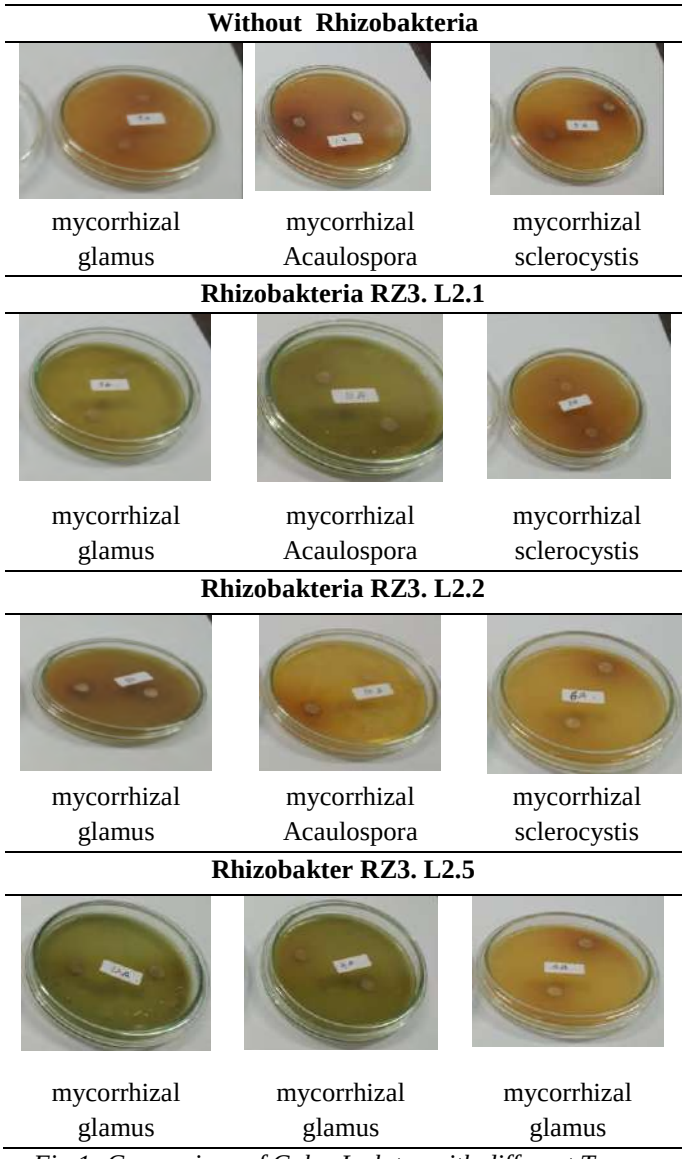

Fig.1: Comparison of Color Isolates with different Types Rhizobakteria and Types Mycorrhizal

While connected to the potato crop, the presence of HCN gas in plant tissue produced by the endophytic bacteria act as a biocontrol environment of plants against weeds, diseases or nematodes. Rizobakteri mechanism as antagonists of pathogens carried by competition for nutrients Fe is also used for other microbial growth. But

the existence of this HCN also appears to increase the growth of plant roots[5].

Other studies have also suggested that the production of HCN one PGPR important properties, these compounds having biocontrol activity and can suppress pathogenic fungi on plant roots. It led to a better condition to root growth for microorganisms contained planting around the plant roots only beneficial microorganisms[6].

2. Infection percentage Roots

Based on Table 2 can be seen that the plants are given rhizobacteria percentage of root infection were higher than without rhizobacteria. Rated highest percentage of root infection inRZ3.L2.2 followed RZ3.L2.5 and RZ3.L2.1. Table 2 also shows that Mycorrhizal glamus have highest percentage of root infection than Acaulospora mycorrhizal and mycorrhizal sclerocystis.

Table 2. Effect of Mycorrhizal Fungi Rhizobakteri and Infectious Fungi against Root Potato at Age 9 Week After Plant

Types Rhizobakteria	Dosis FMA			Average
	mycorrhizal glamus	mycorrhizal Acaulospora	mycorrhizal sclerocystis	
Without Rhizobakteria	37,00	30,00	32,57	33,189 c
RZ3.L2.1	67,33	34,47	52,23	51,344 b
RZ3.L2.2	67,77	61,10	56,67	61,844 a
RZ3.L2.5	61,13	51,10	53,33	55,189 b
Average	58,308 A	44,167 B	48,700 B	
KK =	15,95%			

The figures followed the same lowercase letters in the same column and the same big letters on the same line by DNMRT no significant level of 5%.

If adjusted to the classification of a root infection, then giving treatment rhizobacteria types or species of mycorrhizal root cause infections that are high and on the potato. Like wise, if compared with the Other research then the value of root infection acquired quite high because of infections acquired at the root of Jabon and sweet sorghum is 10%. This condition is caused by a food reserve in Jabon and sweet sorghum is deposited on the rod so that the root exudates which may be used by esearhizobakteri less[6].

Similar results were obtained in other studies, in which the highest root infection is obtained if the plant is given Glamus mycorrhizae. This may be due to the provision of RhizobakteriRZ3.L2.2 and Mycorrhizae glamus have a high N uptake than other treatments. N high nutrient uptake will increase the availability of food needed to increase the percentage of mycorrhizal root infection[7].

Conformity with the requirements of research conditions to grow and proliferate rhizobacteria and FMA also an important requirement for root infection. Increasingly in accordance with the requirements of environmental conditions where the plants grow better plant growth and the higher the percentage of root exudates. Naturally around the plant roots already contains many microorganisms. The type and amount depends on the type of plant. Some types of microorganisms such as FMA have mutually beneficial relationships with plants. The plant is a provider of energy sources while microorganisms infect plant roots thereby increasing the availability of nutrients for plants. The higher the better root infection of plant growth. If the host plant can support the growth and proliferation of FMA well.

3. IAA

Table 3 shows that the plants are given rhizobacteria RZ3.L2.2 contains IAA with RZ3.L2.5 but unlike other treatments. Plants were given Mycorrhizae IAA glamus also contain higher than Acaulospora and Mycorrhizae Mycorrhizae sclerocystis.

Table 3. Effect of Mycorrhizal Fungi Fungi Rhizobakteri and against Total IAA Potato at Age 9 Week After Plant

Types Rhizobakteria	Dosis FMA			Average
	mycorrhizal glamus	mycorrhizal Acaulospora	mycorrhizal sclerocystis	
	µg/ml			
Without Rhizobakteria	11,340	8,903	9,350	9,864 b
RZ3.L2.1	10,797	9,627	10,007	10,143 b
RZ3.L2.2	13,610	10,087	10,600	11,432 a
RZ3.L2.5	11,727	9,563	10,127	10,472 ab
Average	11,868 A	9,545 B	10,021 B	
KK =	7,32%			

The figures followed the same lowercase letters in the same column and the same big letters on the same line by DNMRT no significant level of 5%.

IAA value obtained in this study is quite high when compared to other studies. Other studies show that nine types of rhizobacteria only produce IAA less than 1 ug/ml, 2 types rhizobacteria less than 1.5 ug/ml, 1 kind rhizobacteria less than 3 ug/ml but one kind rhizobacteria 17.72 ug/ml. While IAA obtained in this study is more evenly distributed in the range of grades 9 ug/ml to 11 ug/ml. This is due to rhizobacteria able to fix the roots well. IAA produced will be better when the plant has a root infection are high.

IAA production is influenced by many factors. The ability of endophytic bacteria in producing IAA isolates vary based on the type, age, culture, plants that become hosts and others. IAA synthesis by the microbial dependent pathway of tryptophan where tryptophan used as precursors and taxonomically diverse plant tissue and metabolic different. Some endophytic microorganisms have the potential to synthesize IAA to increase or

stimulate the growth of the event with endophytic colonization[6].

Other research makes it clear that Capacity of rhizobacteria produce IAA is determined by the amount of the amino acid tryptophan provided root plants that can be synthesized by rhizobacteria. This is due to the amino acid tryptophan is a substrate for the formation of the IAA. The ability to produce IAA determined by the type rhizobacteria tested and the ability to colonize plant roots. Rizobakteri's ability to colonize plant roots has implications for the amount of the amino acid tryptophan derived from plant root exudates. IAA production by rhizobacteria will only happen if the concentration of the amino acid tryptophan in the root zone of plants is quite high[7] .

One of the major contributions to the growth of these microorganisms plant is the production of molecules like auxin. 3 indole acetic acid (IAA) into the auxin can stimulate growth such as cell elongation and cell division and differentiation. Compounds Indole Acetic Acid (IAA) is a growth regulator substances classified in hormon and regulates the process of cell growth and development. IAA normally produced in microorganisms through the L-tryptophan. IAA produced by the bacteria around the roots acts as a carrier molecule communication signals between plants and microbes as well as supporting the growth of plants. IAA helps produce root becomes longer by increasing the number of root hairs and lateral roots are involved in decision-nutrition[9].

4. Indeks area index

Table 4 shows that the leaf area index is proportional to the percentage of root infection. Potato plant leaf area index age 9 obtained at the highest MSTmycorrhizal glamus that is 0.499, This may be dueMycorrhizal glamus have a better ability to root infection so that more optimal nutrient absorption and water which is needed in increasing the increase in the number and size of leaves. The treatment of many types of rhizobacteria apparently not affect the rate of assimilation of plants. However, rhizobacteria Award has a value higher net assimilation compared without giving rhizobakteria.

Table 4. Effect Rhizobakteri and fungi Mycorrhizal Fungi on Leaf Area Index Average Average Chips At Age 9 Week After Plant

Types Rhizobakteria	Types FMA			Average
	mycorrhizal glamus	mycorrhizl Acaulospora	mycorrhizal sclerocystis	
Without Rhizobacteria	0,421	0,270	0,312	0,334 b
RZ3.L2.1	0,516	0,491	0,386	0,465 a
RZ3.L2.2	0,526	0,350	0,473	0,449 a
RZ3.L2.5	0,533	0,503	0,442	0,493 a
Average	0,499 A	0,403 B	0,403 B	

The figures followed the same lowercase letters in the same column and the same big letters on the same line by DNMRT no significant level of 5%.

This indicates that the administration of rhizobacteria able to increase crop leaf area index. Although this type of rhizobacteria has its own characteristics, but the same leaf area index. Leaf area index value is closely related to the plant leaf area. Leaf area index is the ratio between leaf area and an area of land that is overgrown potato plants at any time. One of the factors that influence the value of the leaf area index is the number of leaves of the plant. Plants that have a large number also has a size that it leaves the plant will have a value of leaf area index is high[10].

Increased leaf area index contributed positively to the growth of the plant because the leaf is the main organ where photosynthesis. However, the leaf area index value should not be too high and too low. Leaf area index is too high shows many leaves of the plant are inactive photosynthasis. This will inhibit the formation of tubers because the nutrients used by the organ that is not productive. However, the optimum number of leaves that allow the distribution of the light between the leaves evenly. Uniform light distribution across the leaves reduces the incidence of shade each other so that each leaf can cooperate as appropriate. Leaf area index at the beginning of the growth of most plants in the field is zero and for a few weeks and then can be below 1. 0 further increase in leaf area index[11]. Large leaf area is usually maintained until the close before maturity unless the leaves are affected by plant pests and the environment [12].

5. The rate of assimilation Net

Table 5 shows that the potato plant without rhizobacteria with Mycorrhizal glamus had a net assimilation rate equal to the mycorrhizal sclerocystis but larger than Acaulospora mycorrhizae. Rhizobakteri RZ3.L2.1 with Mycorrhizae glamus have the same net assimilation rate with Acaulospora but with a different Mycorrhizae, Mycorrhizae Mycorrhizae Acaulospora sclerocystis but also together with Mycorrhiza sclerocystis. Rhizobakteri RZ3.L2.2 with Mycorrhizae glamus have the

same net assimilation rate with sclerocystis Mycorrhizae Mycorrhizae but larger than Acaulospora. Rhizobakteri RZ3.L2.5 with Mycorrhizae glamus have the same net assimilation rate with sclerocystis Mycorrhizae Mycorrhizae but larger than Acaulospora. However, it can be concluded that all types of rhizobacteria have a high net assimilation rate if the Glamus mycorrhizae.

Table 5. Effect Rhizobakteri and Mycorrhizal Fungi against Net assimilation rate in the Potato Age 9 Week After Plant

Types Rhizobakteria	Types FMA		
	mycorrhizal glamus	mycorrhizl Acaulospora	mycorrhizal sclerocystis
	....mg per cm^2 per week....		
Without Rhizobacteria	0,0071 a A	0,0055 b A	0,0067 a A
RZ3.L2.1	0,0092 a A	0,0054 ab A	0,0014 b B
RZ3.L2.2	0,0062 a A	0,0014 b A	0,0033 ab B
RZ3.L2.5	0,0058 a A	0,0016 b A	0,0051 a A
KK	8%		

The figures followed the same small letters on the same line and the same capital letter in the same column according to DNMRT no significant level of 5%.

Table 5 also shows that Mycorrhizae glamus and mycorrhizal Acaulospora have the same net assimilation rate even with or different types of rhizobacteria. However, Mycorrhizaesclerocystis with RZ3.L2.5 had net assimilation rate were as high as without rhizobacteria but larger than RZ3.L2.1 circuitry. But in general, the greatest assimilation rate obtained on mycorrhizal glamus with rhizobacteria RZ3.L2.1. This indicates that the plant has a value of root infection, leaf area index of the most well also have value net assimilation rate the highest.

The net assimilation rate is the average value of the photosynthetic efficiency of leaves that occur in plants cultivated potato. The highest net assimilation rate obtained when the plants are still small. This is due to the whole leaves of potato plants to get exposure to direct sunlight. Increasing the age of the plant assimilation rate, the value will also increase and the leaves are protected more cause impairment, net assimilation rate. Plants that have a high leaf area index had a chance to absorb the most sunlight, have the highest CO2 assimilation rate and tanslocationlargely the result of assimilation into other parts of the plants[13].

The statement shows that the older the plant, the lower the value of the rate of assimilation of plants. Besides plants have leaf area index values that are too high will reduce the rate of assimilation value plants. The net assimilation rate also affects the reserves translocation of foods derived from metabolic processes[14].

IV. CONCLUSION

The best treatment of this research is rhizobacteria RZ3.L2.2 with mycorrhiza glamus against the net assimilation rate. Rhizobakteri RZ3.L2.2 and mycorrhizal glamus able to increase the value of HCN, the percentage of root infection, IAA content, leaf area index and net assimilation rate.

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Differential responses of exogenous melatonin on growth, photosynthesis and antioxidant defence system in two *Brassica napus* L.cultivars under chromium stress

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Abstract— *Rapid industrialization throughout the world during last few decades causing high chromium resulted widespread of agricultural soil contamination. The increased chromium contents beyond permissible level in some agricultural land areas increasing widespread concern about food safety.This study was carried out for evaluation of metal toxicity damage and its possible mitigation and improved photosynthetic efficiency by melatonin treatment in canola plants exposed to four melatonin levels (0,1,5,10µM) treated with chromium stress (0,50,100µM) for two days.Chlorophyll fluorescence a transients considered one of the best tool for photosynthetic (photosystem II) efficiency analysis of two canola cultivars Ac-Excel and DGL with or without melatonin treatment against chromium stress analyzed by using OJIP test (at different time scale) chromium treated and non-treated plants. Enhanced ROS scavenging antioxidants enzymes (SOD, POD, APX,CAT) and H_2O_2, MD Aactivity photo synthetic efficiency was observed against chromium stress. DGL cultivar showed greatly affected and showed maximum reduction in performing index of photosystem II and yield for primary photochemistry as compared to chromium treated and non-treated plants as compared to Ac-Excel. Performing index primarily comprises of active number of reaction centers as per absorption, primary photochemistry yield and efficiency of electron transfer in electron transport chain activities were observed high in Ac-Excel cultivar. However exogenous application of melatonin protected the oxygen evolving complex of PSII and helped out in maintaining PSII activity. Thus OJIP fluorescence transients are quite helpful for understanding the intersystem electron transport beyond photosystem II response of canola cultivars in chromium stress.*

Findings: *Exogenous application of Melatonin can improve plant growth and development in heavy metal stress by modulation of photosynthesis in terms of enhanced photosystem II efficiency and redox potential in certain environmental stress conditions.*

***Keywords*— OJIP, Chlorophyll fluorescence, Melatonin, chromium stress, Canola.**

I. INTRODUCTION

Heavy metals are naturally occurring trace elements of soil major source of agricultural soil contamination and major threat to food safety in several parts of the world (Ifon et al., 2019). Mostly, metals are found in soils as in the form of insoluble compounds such as oxides or carbonates, metallic complexes and free metallic ions. Among all heavy metals chromium is one of the toxic heavy metal causing severe threat to food security for many developing countries including Pakistan. Excessive use of fertilizers and pesticides, leather tanning, mining, natural disasters including volcanic eruption and weathering of rocks causing high level of chromium contents in agricultural soils (Kotecha et al., 2019). It has been found that chromium affects the physiological processes of plants mainly stunted growth, chlorosis and wilting of leaves, photosynthesis and roots damage, reduction in nutrients uptake ultimately causes death (Aparicio et al., 2019). Plants facing environmental abiotic stress conditions stimulates the formation of reactive oxygen species (ROS) which harms the production of biomolecules including nucleic acids, proteins and lipids, disturbing the sugars metabolisms and mitochondrial respiration of cell.

It has been reported that plants exposed to metal toxicity induces lipid peroxidation, which consequences membrane structure damage, enzymatic and transport activities. However, plant in response activates self-protection mechanism such as cellular enzymatic antioxidants which scavenges reactive oxygen (ROS) and reduces the oxidative stress (Rajendran et al., 2019). The hyperactivity of antioxidants enzymes and their subcellular accumulation in different parts of plants against chromium, zinc, aluminum and copper have been reported in several studies (Ghori et al., 2019). Moreover, it was observed that chromium induced toxicity leads to the poor lamellar system development, fewer grana and enhanced thylakoid lumen ultimately consequences some ultrastructure changes in plant cell (Ali et al., 2013).These changes in ultrastructure of chloroplast might have some negative impact on energy transfer imbalance and photosynthesis.

Furthermore, chromium and other trace elements may cause ultrastructure abnormalities in mesophyll cells and root cells ultimately increased metal deposition in some plant parts. Several previous studies have suggested the inhibitory effect of chromium on PSII activity mostly studies have been performed on cellular membrane damaging effect, to increase knowledge about the negative effect of chromium on photosynthesis PSII activity to evaluate the toxic effect of chromium on photosynthetic apparatus (Souri et al., 2019), Chromium stress induces changes in ultrastructure of chloroplast, electron transport chain (Chen et al., 2019). At PSII chromium ions replaces the co-factor Ca^{2+} known to be very important for water-splitting, hence alters the structure and function of oxygen evolving complex. In addition to oxygen evolving complex chromium ions interacts with many essential electron acceptor proteins i.e Q_B foundin electron transport of PSII (Oves et al., 2016; Küpper et al., 2019).

Primarily melatonin is considered as an antioxidant because of its great potential to control the reactive oxygen species under a-biotic stresses including heavy metal toxicity, salinity, drought, cold and heat stress, ozone stress, chemical pollutants, herbicides and ultraviolet radiations makes it most interesting bio-stimulating molecule for agricultural crops (Kabiri, R. et al., 2018).Several studies have indicated the potential role of melatonin in alleviation of heavy metal stress, salt stress, drought stress, heat stress in many plant species (Wang, L.Y et al., 2015). Melatonin closely associated with reactive oxygen species (ROS) generation and cell signaling under certain environmental stress conditions, thus chloroplast considered the major site of melatonin production (Martinez, V et al., 2018).

Canola (*Brassica napus* L.)is well known and major source of edible oil throughout the world. Brassica species are considered as potential candidate against heavy metal stress because of its distinguishing characteristics such as heavy metal absorption, rapid growth and greater biomass (Meng et al., 2009). Canola plants have developed specific heavy metal tolerance mechanism that enables them to grow well in polluted soil. Thus, it is necessary to evaluate the *Brassica* species response or specific mechanism involved in metal tolerance. Hence, present study was carried out to analyze the *Brassica napus* tolerance against chromium stress by exogenously applied melatonin and its effects on plant growth, chlorophyll pigments, and enzymatic antioxidant system in alleviation of metal toxicity.

II. METHODOLOGY

Plant material and growth conditions present study was carried out to investigate efficiency of PSII in adverse effects of Chromium stress on the growth of *Brassica napus* L. var. AC-Excel and DGL. A pot experiment was performed in agricultural land of Bahauddin Zakariya University,

Multan, Pakistan with normal environmental conditions (30°N and 71°28E). Seeds of canola cultivars were obtained from Ayub Agriculture Research Institute (AARI) Faisalabad. River washed sand was used as a rooting medium. In experiment 120 plastic pots with diameter 28cm with 8 kg sand were used, five to seven seeds were sown in each pot. After germination of the plants thinning was carried out leaving 4 equal distant plants in each pot. After twenty days of germination, plants were treated by various levels of Cr (0, 50, 100 µM) with Hoagland nutrient solution (full strength). After 10 days of chromium treatment foliar application of melatonin with different concentration (0, 1, 5, 10µM) mentioned as MT0, MT1, MT2, MT3 were applied exogenously to the plants. Experiment was designed according to CRBD (completely randomized block design) with three Cr levels and four melatonin levels, two cultivars and five replicates for each treatment. After twenty days of treatment, plant fast chlorophyll a kinetic analysis was measured with Fluor Pen. After taking data of all parameters mentioned above, plants were harvested. Plants root and shoots were separated. Roots were washed out and plant fresh biomass (root and shoot) were measured. For dry biomass samples of plants (root and shoot) were dried in oven at 70 °C for 48 hours, then samples weight taken in grams (g) by digital electronic balance.

Chlorophyll contents:

For the estimation of chlorophyll contents of canola plants 0.2 gram leaf tissue was taken homogenized in 80% acetone in pestle and mortar. Extract after filtration was kept 10 ml volume by adding 80% acetone in falcon tubes wrapped by aluminum foil to prevent chlorophyll degradation in light. Chlorophyll contents were measured at different wave lengths 663,652,645, and 470 nm by usingspectrophotometer (U-2900/2910 Hitachi).(Arnon, 1949).

Analysis of O-J-I-P fast chlorophyll a transients

Chlorophyll fluorescence data was recorded following nomenclature by (Kodru *et al.*, 2015) and literature related to chlorophyll fluorescence available on its manufacturer website. For this fully matured third leaves of canola plants were selected, by using hand held device Fluor Pen FP 100fluorescence transients were observed by keeping plants in dark by using aluminum foil for 30 minutes.

Antioxidants enzymes activity

Fresh plants leaf tissue of 0.1 gram was homogenized in pre-chilled pistal and mortal with 1% (w/v) polyvinyl poly pyrrolidone solution with 1.2 ml of 50mM potassium phosphate buffer by maintaining pH 7.8 along with adding 1mM EDTA-$Na_2$and 0.3% Triton X-1000 solution.

APX enzyme activity was estimated by adding 1mM ascorbate solution to prepared solution, Extract was centrifuged at 13,000 rpm for 20 minutes maintaining temperature at 4°C extract was used for following enzymes activities. SOD activity was measured according to (Zhang et al., 2013) methodology. Reaction mixture of 3 ml including 13mM methionine, 75mM NBT, 2mM riboflavin, 0.1mM EDTA and 100µL enzyme extract along with 50mM sodium phosphate buffer (pH 7.8).Reaction mixture was illuminated at light intensity of 90 for about 25 min µmol/m^{-2} s^{-1}. SOD activity was observed by measuring the enzyme extract ability or activity (µmol min^{-1} g^{-1}) of photochemical reduction of NBT (about 50%) by using spectrophotometer at 560 nm.CAT activity (µmol min^{-1} g^{-1}) was observed by reduction in absorbance of reaction mixture at 240 nm by decomposition of H_2O_2in 1 ml of reaction mixture with 50mM sodium phosphate buffer (pH 7.8) in addition 10mM H_2O_2, 20 µl of enzyme extract according to (Aebi 1984).

POD activity (µmol min^{-1} g^{-1}) was observed by preparing 1 ml reaction mixture with 100mM sodium phosphate buffer (pH 6.0) 16mM guaiacol solution 5µl of 10% H_2O_2(w/v) solution by following (Rao *et al*. 1996).APX activity (µmol min^{-1} g^{-1}) was observed by reduction in absorbance at 290 nm as reduced ascorbate was oxidized in 1 ml of reaction mixture containing 50mM hepes-KOH of 7.6 pH with 0.1mM EDTA, 0.5mM ascorbate, 0.2mM $H_2O_2$and 20µL enzyme extract, reaction was started by the addition of H_2O_2(Nakano and Asada 1981).Glutathione reductase activity was observed by following the methodology of (Griffith 1980). Fresh leaf tissue 0.1 gram were homogenized in pre-chilled pistil and mortars in 1.5 ml of 5% sulfosalicylic acid and centrifuged at 12000 rom for 15 minutes then absorbance of supernatant was used at 412 nm for measurements.

Estimation of MDA and H_2O_2

H_2O_2contents were measured by 5% trichloroacetic acid solution by following (Zhou *et al.*, 2006). MDA were measured according to the method of (Hodges *et al*., 1999).

Estimation of chromium (Cr)

For the determination of chromium contents 0.1g of dried leaf samples were taken in digestion flask with 2ml of digestion mixture was mixed and kept pre-night for about 12

hours for complete digestion of leaf plant tissue in digestion mixture. After that flasks were heated on hotplate by gradually increasing the temperature from 50^0C to 200^0C. By heating, color of plant samples turned black, at this stage about 0.5ml of $HClO_3$ was added by using dropper. After this by increasing temperature plant samples become transparent. Then flasks were taken off from hotplate, cooled and diluted by adding 50ml of distil water. Then the calculation of chromium contents were performed by using atomic absorption spectrophotometer.

III. RESULTS

Chromium toxicity causes reduction ($P \leq 0.001$)in biomass (fresh and dry weight g/plant) (Fig.1) and Leaf number and quantum yield (Fig 2) of both the canola plants that of control plants. While melatonin treated plants showed improved plant growth in terms of fresh and dry biomass and leaf number in addition to quantum yield of both canola cultivars, especially at 5µM concentration melatonin treated Ac-Excel cultivar showed significant increase in plant height, leaf number and biomass as compared to DGL cultivar with and without chromium stress.

Overall chlorophyll contents were significantly affected by chromium stress ($P \leq 0.001$) including chlorophyll a, chlorophyll b, chlorophyll a/b and total chlorophyllof canola plants (Fig.3). While exogenous application of melatonin improved chlorophyll contents meanly 5 and 10µM concentration significantly increased chlorophyll contents in chromium treated and non-treated plants. Significant increase in plant height, biomass and chlorophyll contents in Ac-Excel shows more resistance as compared to DGL in chromium stress.

Whereas plants antioxidants activity was observed to be significantly higher ($P \leq 0.001$) under chromium stress as compared to control plants in both melatonin treated and non-treated canola plants. Chromium toxicity leads to enhance the total soluble proteins contents and reactive oxygen species H_2O_2 and MDA (Fig 4) consequently more production of ROS scavenging enzymes such as superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT) to minimizechromium toxicity (Fig 5), additionally canola plants showedhigh APX (Ascorbate peroxidase) and Glutathione reductase enzyme activity (Fig 5) to scavenge/lower H_2O_2or oxidative stress due chromium stress.

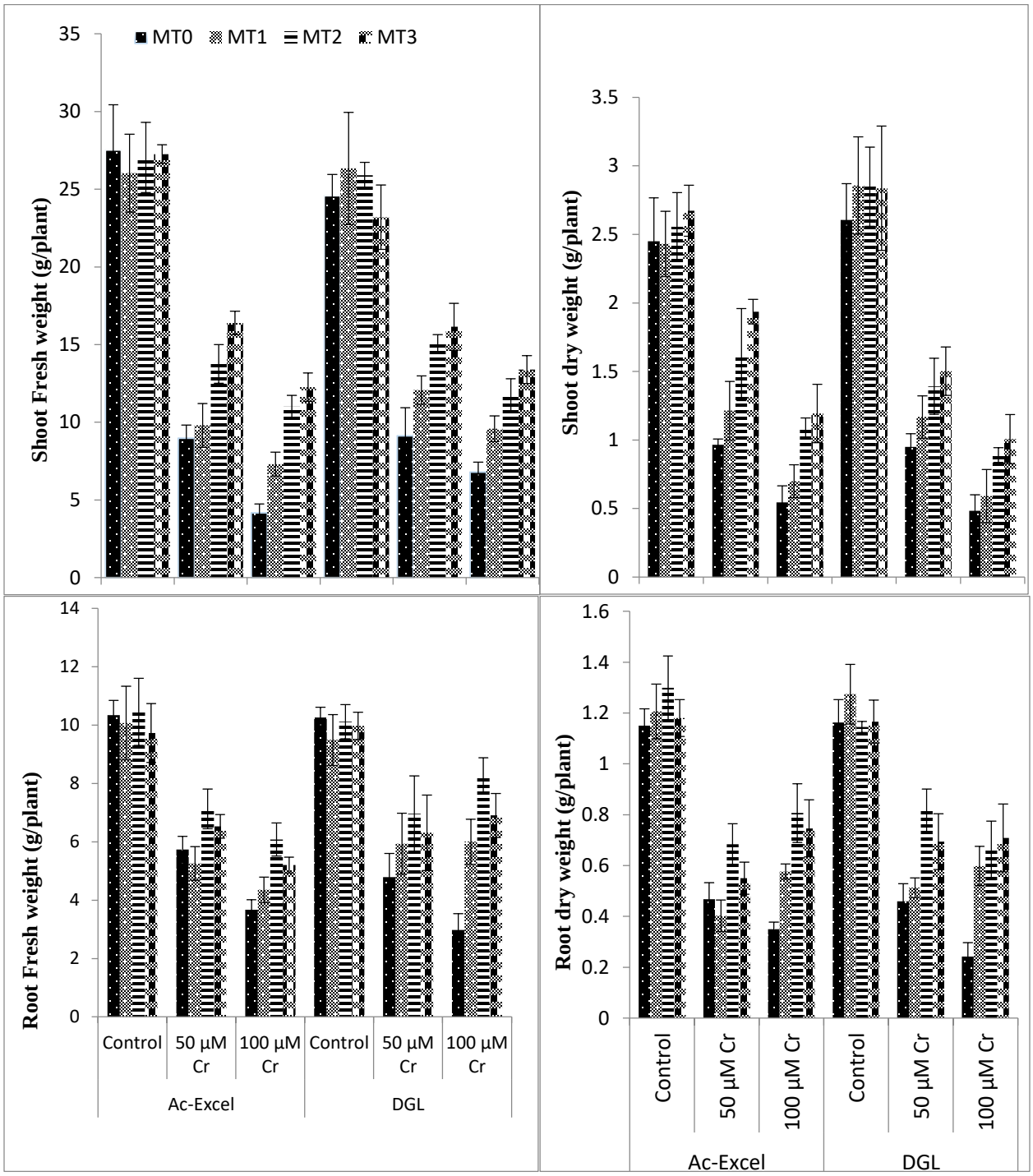

Fig.1: Melatonin induced Biomass changes of two Canola plants treated with chromium stress for two weeks.

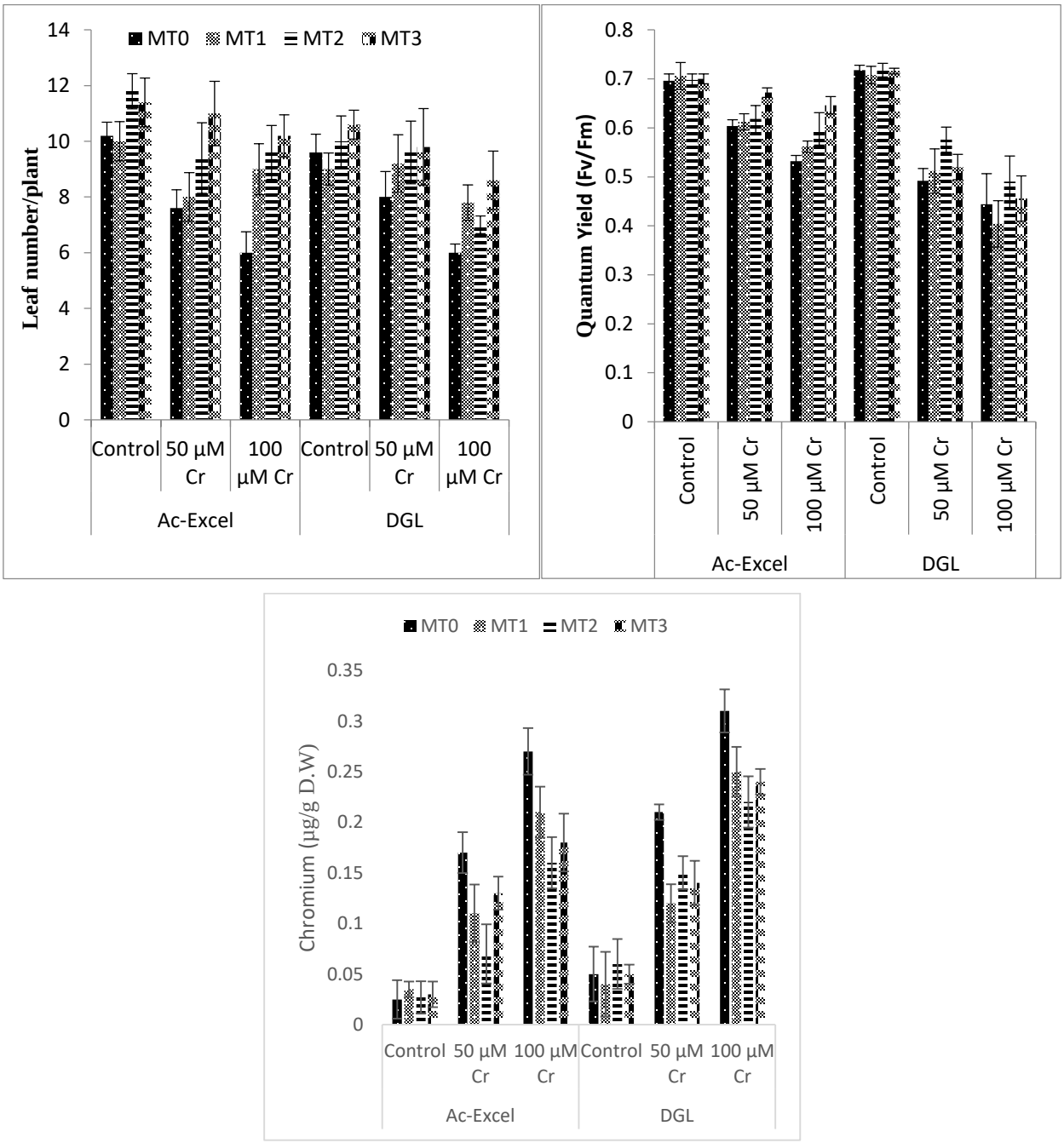

Fig.2:Melatonin induced Leaf Number/plant, Quantum yield and Chromium contents of two Canola plants treated with chromium stress for two weeks.

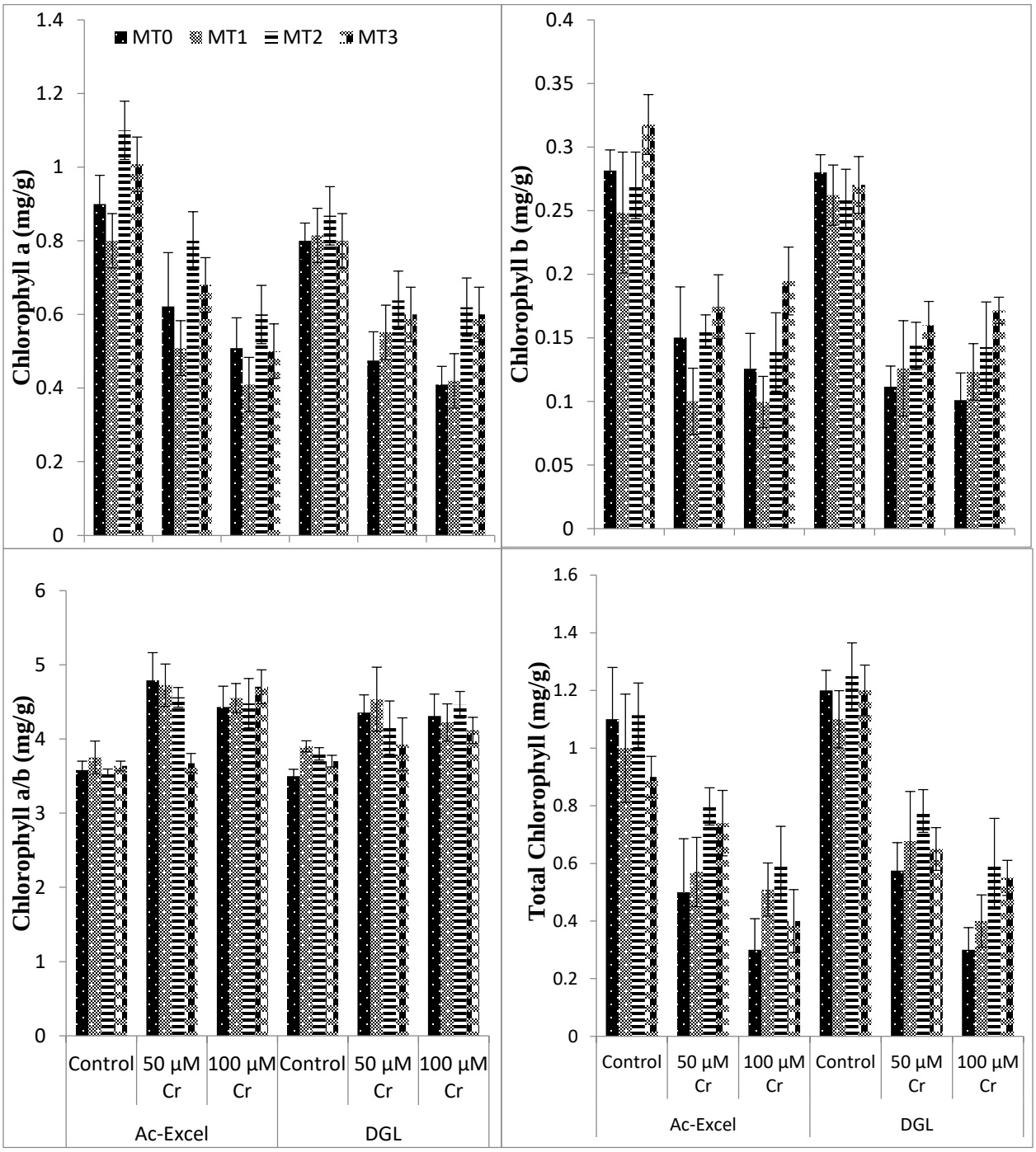

Fig.3: Melatonin induced Chlorophyll contents (mg/g) of two Canola plants treated with chromium stress for two weeks.

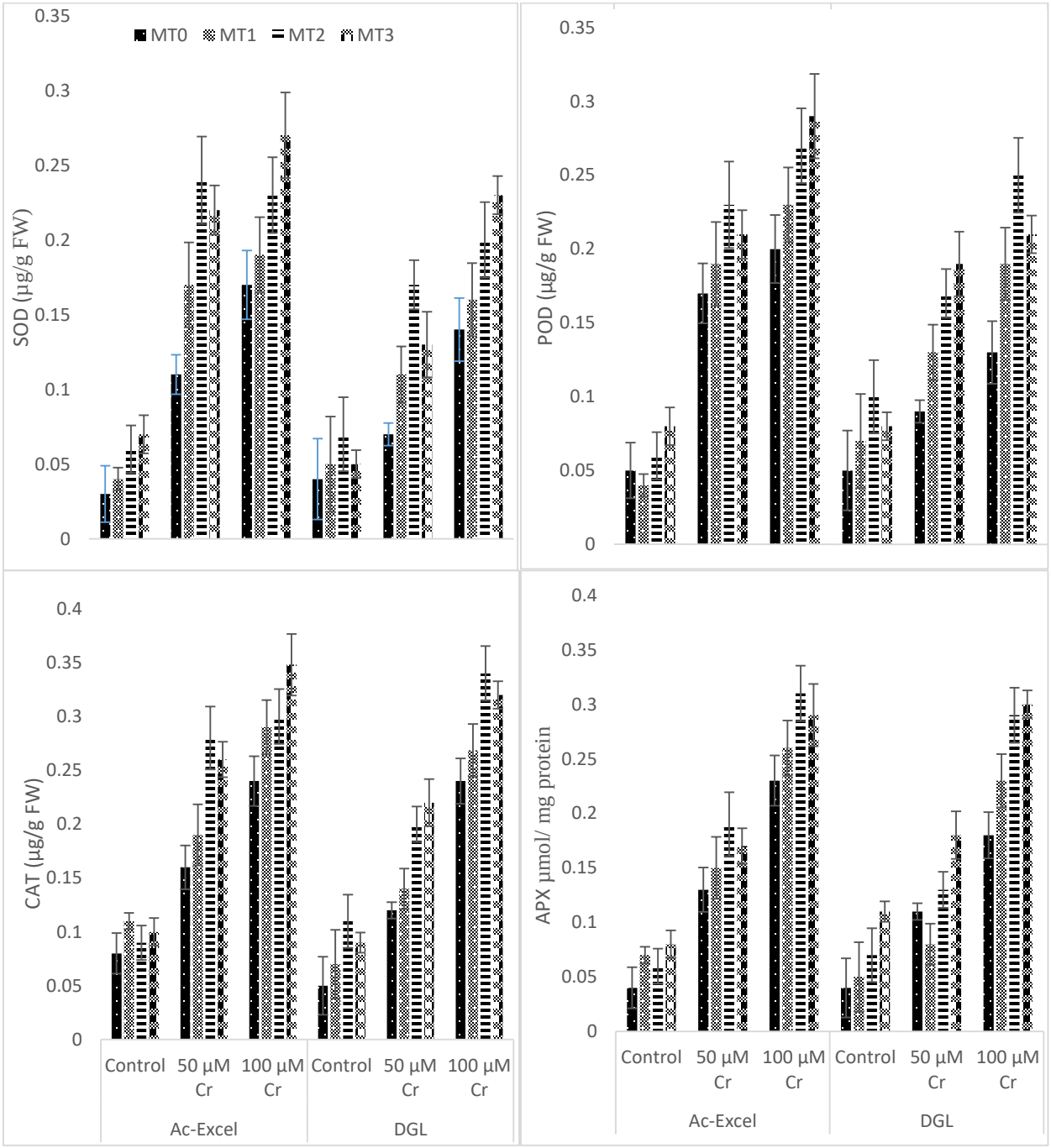

Fig.4: Melatonin induced SOD, POD, CAT, APX of two Canola plants treated with chromium stress for two weeks.

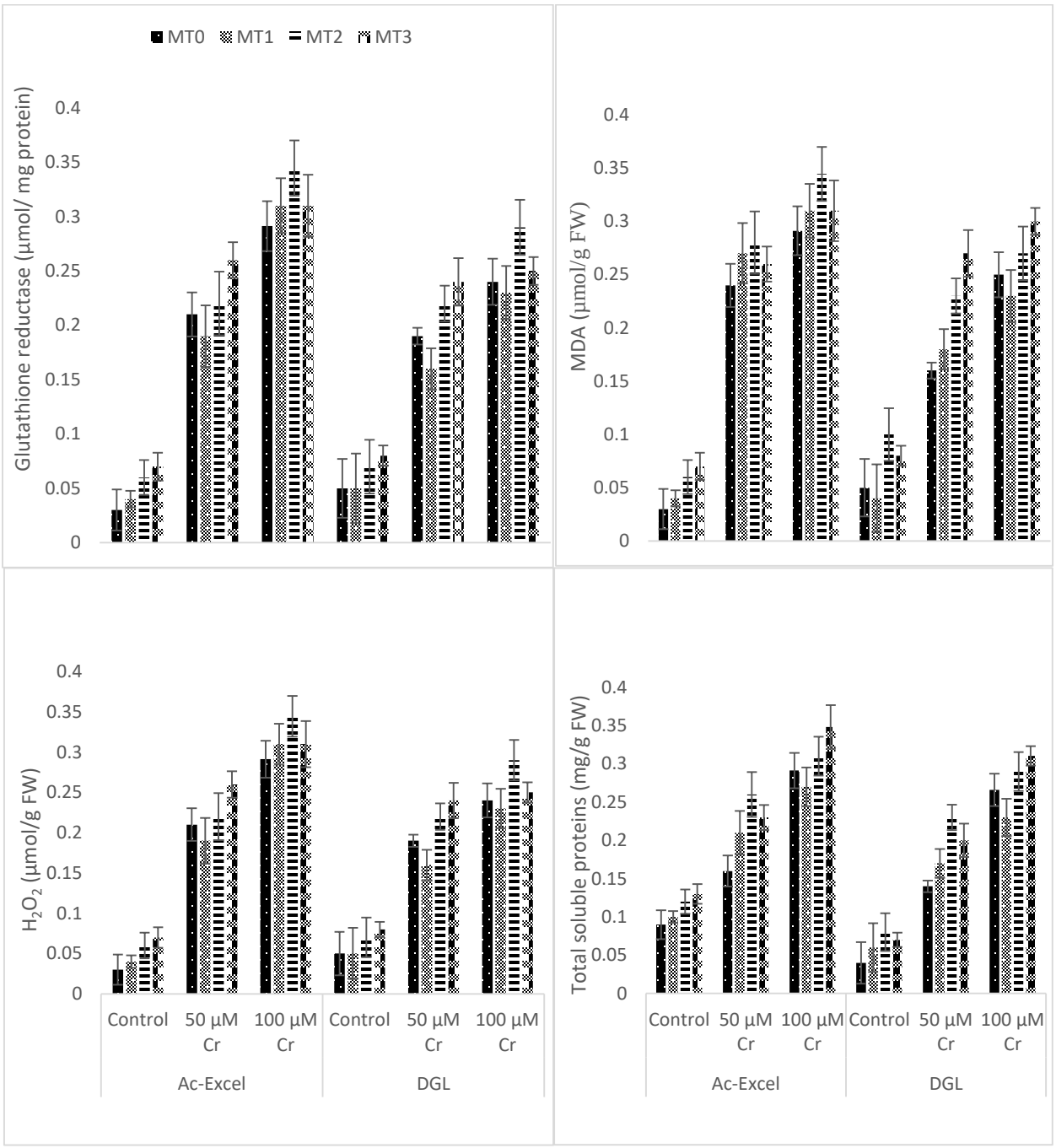

Fig.5: Melatonin induced Glutathione reductase, MDA, H_2O_2, and Total Soluble Proteins of two Canola plants treated with chromium stress for two weeks.

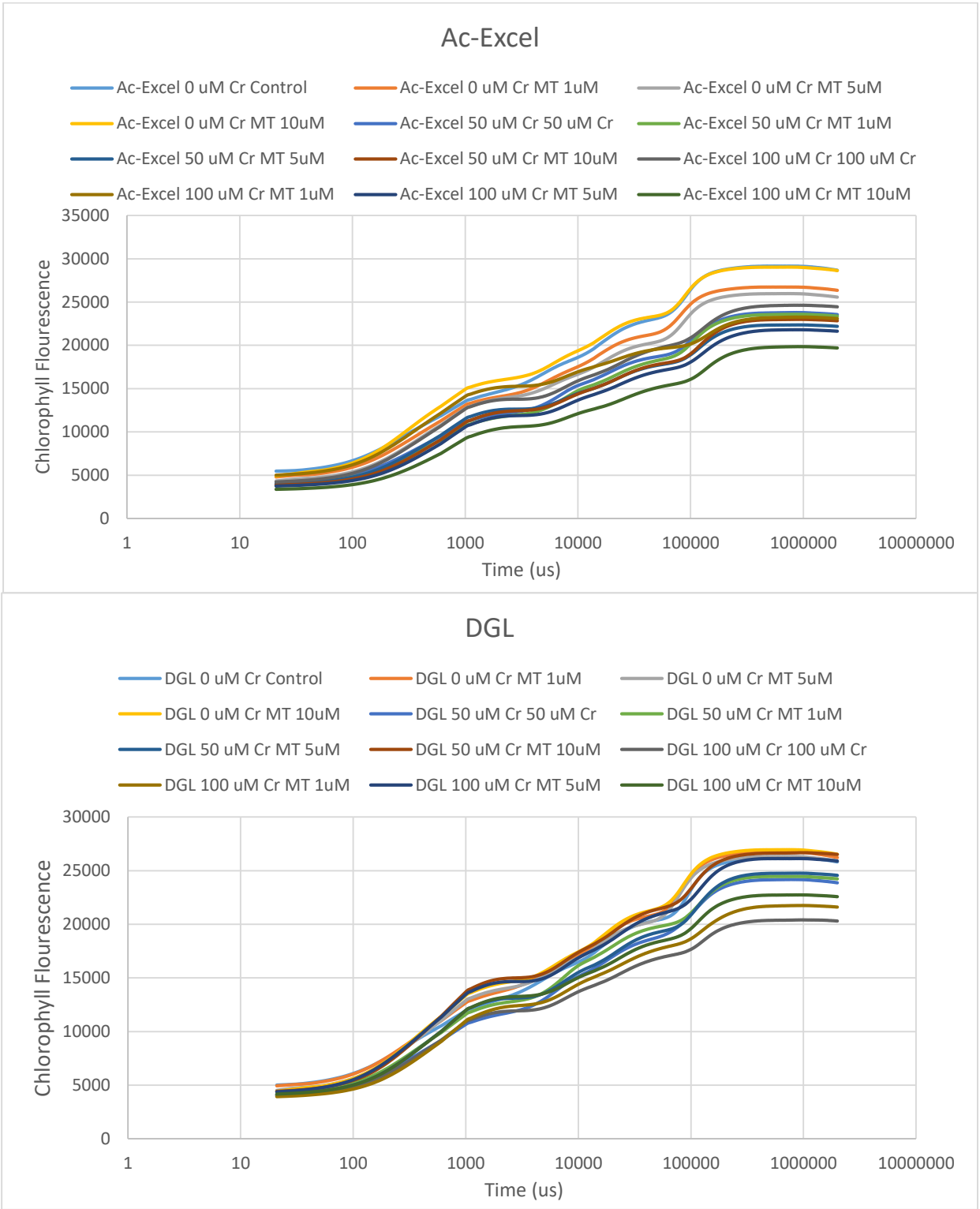

Fig.6: Melatonin induced Chlorophyll fluorescence variations of two Canola plants treated with chromium stress for two weeks.

However, melatonin treated plants showed high antioxidants enzymes activity maximum activity was observed at 5µM concentration in chromium stress as well as in control conditions. This special increase in antioxidants were higher in Ac-Excell that of DGL cultivar.

Suggesting that Ac-Excell has higher potential and tolerance of metal toxicity by showing significantly more ROS contents i.eH_2O_2 and MDA and antioxidants enzymes such as superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT), APX (Ascorbate peroxidase) and Glutathione reductase enzyme activity.

Chromium contents were observed significantly higher in chromium treated plants in both the canola plants, but cultivar DGL showed higher chromium accumulation that of Ac-Excell cultivar suggesting that hyper accumulation of chromium shows more damaging effect on plants physiology or overall plant growth that of Ac-Excell (Fig 2).

Foliar application of melatonin increased the amplitude of I-P curve in both genotypes of canola under control conditions, while in chromium stress amplitude is lowered in both the cultivars. Suggesting that melatonin may play vital role in increasing the electron pool carrier of photosystem I end, to be reduced from electron coming from PQ in both canola cultivars. Whereas I-P band that is measured as VIP=[(Ft-FI)/(Fm-FI)] indicates that chromium stress reduces the rate of constant value of Ac-Excell melatonin enhances the rate of constant value in Ac-Excell cultivar that of DGL. Chromium stress induced biophysical changes derived from chlorophyll fluorescence curve of canola cultivars explained as in radar plot as shown in (Fig.6).

However, improved values of Fv/Fo and Fm/Fo because of melatonin treatment was observed more in Ac-Excell under chromium treated that of non-treated plants. While increased values of Mo (primary photochemistry values) in Ac-Excell cultivar was observed to be improved in foliar application of melatonin that of chromium treated plants of canola shown in (Fig.6). Similarly, total Area (PQ pool), redox state of multiple PQ turnover (Sm) and Q_A redox turnover until Fm actually (N values) was observed to be decreased in DGL only and melatonin application did not significantly affect the biophysical parameters as shown in (Fig.6). The derived fluxes of specific energy including ABS/RC (absorbance flux per reaction center), TRo/RC (trapped energy flux per reaction center), ETo/RC (electron transport flux per reaction center) and DIo/RC dissipation energy flux per reaction center all of these fully reduced in chromium stressed plants of both cultivars and melatonin treatment induced improved OJIP transients in cultivar Ac-Excel cultivar then DGL this rapid electron transfer (reduction) rate becomes faster due to chromium toxicity, because of inactive reaction centers that of control plants.

In this regard our results suggested that exogenously applied melatonin under chromium stress have higher capacity to convert light energy to chemical energy which can be used to further CO_2 to carbohydrates. Conversion of light energy into chemical energy was observed to be higher in cultivar Ac-Excell then DGL.

IV. DISCUSSION

To alleviate the chromium stress induced reduction of canola cultivars, foliar application of melatonin is considered to be one of most affective strategy (Farouk and Al-Amri, 2019) and it has been confirmed in this study. Considerably, exogenously applied melatonin in addition to endogenously melatonin increases chromium tolerance and plants antioxidants defense capacity at significant level. We supposed that melatonin induced chromium tolerance and antioxidant defense system (ROS scavenging mechanism) by production of phyto-chelatins and compartmentalization of chromium in cell wall and vacuole plays a key role in chromium tolerance for canola plants (Roychoudhury *et al.*, 2012).Similarly in the following study melatonin induced increased SOD,POD,CAT,APX and GR activity, which might modulates plants antioxidants activity by inducing ROS scavenging activity (lowering oxidative stress) against chromium stress (Fig 4,5).As melatonin treatment can decrease chlorophyll degradation, increased photosynthesis, antioxidants ability and drought tolerance cucumber seedlings (Zhang *et al.*, 2013). It is assumed that melatonin induced photosynthetic ability in plants is because of some unusual bio-stimulating pathway by modulating photosystem II efficiency in certain light and dark conditions (Zhao *et al.*, 2019).

Inside plants metal toxicity can be reduced by their reactions with metal ligands such as proteins, polysaccharides and organic acids (Andresen *et al.*, 2018) until ratio of non-chelated metallic ions changed into metabolic organelles such as nucleus, chloroplast and mitochondria. These freely available metallic ions causes severe damage to these cellular organelles. Several previous studies suggested that metallic ions sequestration in root

cortex and endodermis occurs because of decrease in transportation of metallic ions (acts as ultimately effective barrier) from root to shoot of plants (Song *et al.*, 2017). In our study exogenously applied melatonin treated plants showed decreased transportation of chromium contents in cell wall and vacuole consequently reducing the chromium toxicity suggesting that melatonin acts as barrier by reducing transportation of chromium in 50 and 100µMchromium treated canola plants. Metallic ions (chromium ions) immobilization assumed to be co-related by melatonin induced biosynthesis of thiol compounds.

Metallic ions competes with mineral nutrients for the same transport system from root to shoot resulting ionic imbalance and disturbed plasma membrane stability (Nazar *et al.*, 2012). H^+-ATPase of plasma membrane that are responsible for the translocation of organic compound and ions across the plasma membrane (Gévaudant *et al.*, 2007).Possibly melatonin improves this transportation of H^+-ATPase by its conversion into 5-methoxytryptamine that stimulates H^+-ATPase activity in addition protects plasma membrane by reducing reactive oxygen species generation and enhancing antioxidants enzymes activity(Jiang & Zhang, 2003).

Accordingly, in our study improved membrane stability, ions transportation and chromium tolerance in melatonin treated canola plants might be due to improved H^+-ATPase activity in chromium treated plants.

Chromium toxicity affects plant photosynthesis process at very large scale, whereas Fv/Fm usually acts as key indicator of plant photosynthesis ability of plant. Generally Fv/Fm always verified as a result of different pigment concentration and cell structure and can be affected by several environmental factors i.e light, nutrients, temperature and certain chemicals that alters the PSII efficiency (Li et al., 2019). Several studies explained that chromium toxicity alters the structure and function of reaction centers and effects electron transport system which consequently reduces the Fv/Fm. Furthermore our results suggested that chromium toxicity significantly alters the PSII efficiency. Melatonin application in chromium stress prevents pigment degradation that helps in improving the overall photosynthetic process. Plants exposed to metals in root region causes inhibition of growth by producing reactive oxygen species that ultimately leads to plants death (Mizushima et al., 2019).

In such conditions plant increases the endogenous melatonin biosynthesis to cope up the metal toxicity as pea plants alleviates the copper stress (Ren et al., 2019). Several studies focused on the phytoremediation ability of plants by exogenous application of melatonin exposed to metal stress by enhancing root growth, antioxidant activity, photosynthesis, by organic acid anion exudation, by reducing metal contents, by increasing antioxidants related gene expression (Arnao and Hernández-Ruiz, 2019; Zhang et al., 2019) and by reactivating the micro RNA mediated redox homoeostasis in different crops (Wang et al., 2019). Similarly, exogenous application of melatonin with 150 µmol/L for eggplant was considered as best concentration for plant against cadmium stress. Melatonin enables plants in cadmium sequestration and transformation from cytosol to vacuole and cell wall (Lv et al., 2019). In addition melatonin application mitigates heavy metal stimulated oxidative stress by enhancing enzymatic and non-enzymatic antioxidant activity (Kaya et al., 2019). Whereas melatonin has amazing efficiently to up regulate the ion channel expression against cadmium stress. However, melatonin with 1µmol concentration treatment alleviates the boron toxicity by improving nutrients uptake efficiency, photosynthetic activity, carbohydrates accumulation, antioxidant defense system and reduces reactive oxygen species (ROS) and membrane permeability in winter wheat (Qiao et al., 2019).

However, melatonin induced improvement in photosynthetic efficiency of Ac-Excel cultivar that of DGL is because of its genetic potential but its effect on the exact site of photosynthetic apparatus is still unclear. In a semi-quantitative observation of melatonin treatment with and without chromium stress on different parts of photosynthetic apparatus of canola cultivars, whole OJIP normalized transients of chromium stressed and non-stressed plants were measured. All the transient data of canola plants of present study explained that primary photochemistry fluorescence and photo electrochemical quenching at O-J and J-I step reduced due chromium stress in both canola cultivars whereas melatonin application increased the photosynthetic activity by compensating reduction rate at PSI and electron acceptor at step I-P site in Canola cultivars especially in Ac-Excel than DGL. In addition Fo normalized transient and relative variable fluorescence transients of Fo and Fm verified our results (Fig.6), for detailed analysis whole difference of kinetics at each step from OJ-JI-IP was performed (Fig.3, 4, 5). Low fluorescence values in L-band in chromium stress conditions showed that loss of energetic connectivity to some extent due to chromium toxicity.

Similarly, K-band showed both canola cultivars showed maximum ability of resistance for donor and acceptor sides of PSII imbalance at 1000 µs against chromium stress. While, an increase in K-band peaks from 1000-2000 µs showed reduced oxygen evolving complex (OEC) performance because of electron flow imbalance from (OEC) to reaction center at acceptor site of PSII in chromium stress. However, at 1000-2000 µs decreased fluorescence curve at K-band under melatonin treatment against chromium stress (Fig.4, 5) suggested that both the canola cultivars showed maximum resistance to electron imbalance at donor and acceptor sides of PSII. Similarly, at O-I step (describes redox properties of PQ poll) decreased/negative fluorescence (ΔVOI) curve described the involvement of melatonin in maintaining the maximum PQ reduction rate in both cultivars against chromium stress.

Meanwhile, fluorescence curve at I-P step that indicates the electron transfer rate from $PQH_{2\ to}$ electron accepter end of PSI, melatonin treated plants showed positive increased in fluorescence transient values at I-P step in chromium stress, while decreased in chromium treated plants suggesting that exogenous melatonin application enhanced PQ redox rate ultimately lowering the chromium stress and increased PQ pool size in both the canola cultivars. Decreased fluorescence transient curve at I-P phase eventually happens because of sharp decline of leaf water status in chromium stress that might reaches to maximum tolerance level of pants. Chlorophyll fluorescence transients and their different ratios at each step of OJIP considered as key indicators for PSII efficiency evaluation.

Chromium stress significantly reduces Fo (minimum fluorescence level) that eventually increases energy excitation and transfer rate from antenna complex to reaction center ultimately leads to low Fo. However, melatonin treated plants also have reduced Fo values, resulting increase in electron transfer efficiency from antenna complex to PSII reaction center. While Fm (maximal fluorescence) values were also reduced in chromium stress that explains the reduction in electron transfer to PSII acceptor site, indicating induced changes in Q_A reduction rate. Foliar application of melatonin enables the plants to maintain balance of plastoquinoneredox state by transferring electron to PSI. Melatonin treated plants showed reduction in ABS/RC values suggested, increased size of antenna complex of active reaction centers. However, PI most sensitive parameter of OJIP indicates the conformational changes and confirms the vitality canola plants of PSII. While exogenously applied melatonin in chromium stress increased the PI values and possible link between ETo/RC and log PI_{ABS} that suggests the utilization of PAR which reduces the CO_2 into sugars in natural environmental conditions.

V. CONCLUSION

The melatonin induced difference observed between two canola cultivars suggests that cultivars have different metal tolerance capability in chromium stress. Chromium toxicity reduced the plant growth, chlorophyll contents and photosynthetic activity in both the cultivars but DGL showed greater effect indicating more sensitive as compared to Ac-Excel. Similarly, enzymatic antioxidant activities were increased in Ac-Excell cultivar suggesting the greater metal tolerance and photosynthetic response against chromium stress. To overcome these stressful conditions exogenous application of hormones as melatonin used in our study effectively can increase the plant growth, development and tolerance against certain environmental stress. There is need to focus on exogenous application of growth enhancing agents that enables plants especially agricultural crops to increase their yield and tolerance against toxic elements.

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The Influence of Shearing Stress on Thermal Homeostasis and Performance of Barki Ewes in the North Western Desert of Egypt

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Abstract**—***Twenty four adult non-pregnant; non-lactating Barki ewes, aged 3 to 4 years with initial body weight of (35.75 ± 1.15) (kg) were randomly divided to two groups (n=12). Ambient temperature (AT) and relative humidity (RH) were recorded along the trail and temperature humidity index (THI) was estimated. The first group was kept as a control unshorn group while the second group was shorn to inspect the effects of shearing on body weight, physiological responses and thermal homeostasis of shorn ewes. Animals were weighed on days 0, 15, 30 and 45. Rectal temperature (RT), skin temperature (ST) and respiration frequency (RR) were measured on days 0, 1, 3, 15, 30 and 45 andThermal gradients (RT-ST) and (ST-AT) were assessed.*

Irrespective of the lost fleece weight on day 0, shorn ewes continued to lose more of their weights till day 15 then they started to regain their body weight. Shearing reduced (P<0.05) RT and RR of shorn ewes to lower values than their control mates. It also declined ST of shorn ewes (P>0.05) compared with the control group. Differences in (RT-ST) and ambient (ST-AT) between groups were insignificant. Shorn ewes retained lower values of RT, ST and RR than the controls during the experiment.

Results indicated the capacity of shorn Barki ewes to maintain their thermoregulation stability by using energy retention mechanisms andto regain body weight. That might indicate a temporal moderate effect of shearing if it was under mild climatic conditions.

Keywords**—** ***Barki, Ewes, Homeostasis, Shearing, Stress***

I. INTRODUCTION

Wool fleece helps sheep maintaining its thermal balance under hot and cold climates as it resist thermal exchange between sheep body and the ambient atmosphere. Consequently, it helps in maintaining core temperature, skin temperature and respiration rate of sheep [21].

Shearing is a routine work in the different sheep rearing systems to harvest wool. In extreme environments, shearing modifies the magnitude and direction of heat exchange and shifts the thermo-neutral zone that impeding the maintenance of homeothermy [4].

The physiological responses of sheep differ according to the surrounding climatic conditions of the shearing season [26], [27] and [28]. Irrespective of season, shearing is associated with some degree of thermal stress that might affect the welfare and productivity of sheep [4] by altering its thermal balance[21] and shifting adaptive thermogenesis via nervous responses to readjust energy saving mechanisms related to climatic adaptability [2], [8] and [20].

Traditionally, this process takes place during spring to avoid the negative effects of climatic extremes during hot and cold seasons. However, spring shorn sheep may be exposed to a wide range of climatic variations due to the fluctuations of ambient temperature and relative humidity rather than the observed differences between day and night temperatures during this season. Even under mild climates, shearing corrupts heat balance mechanisms as it makes sheep more reliable to climatic stress after removing its insulating layer, fleece. Therefore, several changes could be expected in rectal temperature, skin temperature and respiration frequency [8], [23]and [27].The impact of shearing as stress stimulus ranges between moderate [11] to effective [12] stressor.

The current study aimed to evaluate the effects of shearing stress on body weight, physiological parameters and thermal homeostasis of Barki ewes reared under the

semi-arid desert conditions of the northwest coastal belt of Egypt.

II. MATERIALS AND METHODS

This study was carried out in Maryout Research Station (32º N Latitude, 35 km southwest of Alexandria), Desert Research Center, Ministry of Agriculture and Land Reclamation, Egypt. This location represents the semi-arid desert conditions of the northwest coastal belt of Egypt.

2. 1 Meteorological data

Maximum, minimum and average ambient temperature (AT, ºC) besides relative humidity (RH, %) were recorded daily along the experiment by data logger (Gemini. Chichester, UK).The amplitude between the maximum day and minimum night temperatures was calculated by subtraction (ºC).Temperature-Humidity Index (THI), as an indicator of thermal comfort of sheep, was calculated according to Casella *et al.*, (2016) as follow:

THI = AT - 0.55 (1- (0.01 RH) (AT-14.5).

Where AT: average ambient temperature (ºC) and RH: relative humidity (%).

2. 2 Animals and management

Twenty four adult non-pregnant, non-lactating Barki ewes, aged 3-4 years with average initial body weight of 35.75 ± 1.15kg were randomly divided to two equal groups (n=12). All animals were apparently healthy and free of internal and external parasites. The first group was kept unshorn to serve as control while the ewes of the second group were shorn in spring (27th April, 2018). Shearing was conducted manually at the same time for all the shorn ewes and lasted for about 5 minutes for each head. The remained wool after shearing was of average length of 0.5 cm.

Animals were housed in sheltered semi-open pins and fed concentrate feed mixture (0.5 kg head-1 day-1) consisted of 50% cottonseed cake, 15% yellow corn, 18% wheat bran, 11% rice polish, 3% molasses, 2% limestone and 1% common salt. The concentrate mixture contained 60% TDN and 14% CP. Berseem hay (*Trifolium alexandrinum*) was offered *ad.Lib*., drinking access was available twice a day.

2. 3 Live body weight

Live body weight of the experimental animals was recorded in early morning before feeding and drinking to the nearest 0.1 kg by digital balance. On the day before shearing (day0), all animals were weighed twice before and after shearing. The weighing process was repeated on days 15, 30 and 45 after shearing.

2. 4 Physiological parameters

Rectal temperature (RT, ºC), skin temperature (ST, ºC) and respiration frequency (breath/minute) were measured on days 0, 1, 3, 15, 30 and 45, thereafter. Rectal temperature (RT) was measured to the nearest 0.1 °C using a standard clinical thermometer inserted into the rectum. Skin temperature (ST) was taken using a digital thermometer placed over the skin at the mid-side regions of each ewe. Respiration frequency was recorded by counting frequency of flank movements per minute; all required precautions were considered to avoid animal's disturbance.

2. 5 Statistical analysis

The effects of treatment, time, day and all the possible interactions among the aforementioned factors on the studied parameters were examined by illustrating the experimental animals in factorial design trail. Least square means and standard errors of experimental groups were calculated by using proc. GLM of SAS (2013) program according to [25]. Differences between means were estimated by Duncan multiple tests at confidence level of 0.05 for independent factors and their interactions according to the following statistical model:

The effects of treatment, time, day and all the possible interactions among the aforementioned factors on the studied parameters were examined by illustrating the experimental animals. Least square means and standard errors of experimental groups were calculated by using GLM Proc. of SAS (2013) program. Differences between means were estimated by Duncan multiple tests at confidence level of 0.05 for independent factors and their interactions according to the following statistical model:

$$Y_{ijkl} = \mu + T_i + M_j + D_k + (T_i \times M_j) + (T_i \times D_k) + (M_j \times D_k) + A_l\,(T_i) + e_{ijkl}$$

Where:

Y_{ijk} : Any observation,

μ :Overall mean,

T_i :Effect of i^{th} treatment (i = 1-2),

M_j: Effect of j^{th} time (j = 1-2),

D_k: Effect of k^{th} day (k =1-6).

$T_i \times M_j$: Effect of interaction between i^{th} treatment and j^{th} time,

$T_i \times D_k$:Effect of interaction between i^{th} treatment and k^{th} day,

$M_j \times D_k$: Effect of interaction between j^{th} time and k^{th} day,

$A_l\,(T_i)$: Effect ofeach l^{th} animal within i^{th} treatment (as error 1).

e_{ijkl} :residual (as error 2).

III. RESULTS AND DISCUSSION

3. 1 Meteorological conditions

Meteorological measurements were summarized in (Fig. 1). Average ambient temperature changed within a narrow range of about 4 ºC where it tended to increase on day 45 to its highest value. In contrast, the amplitude between day and night temperatures declined on days 30 and 45 indicating an increased night temperature in this period. Relative humidity increased gradually by about 2.5 % from day 0 to day 15 and then it increased sharply in day 30 (about 5%) to reach the highest record at day 45 (72%). Temperature-humidity index (THI) did not seriously differ along the experiment and were within the comfort zone for sheep as THI values were <72 values along the experiment[1].

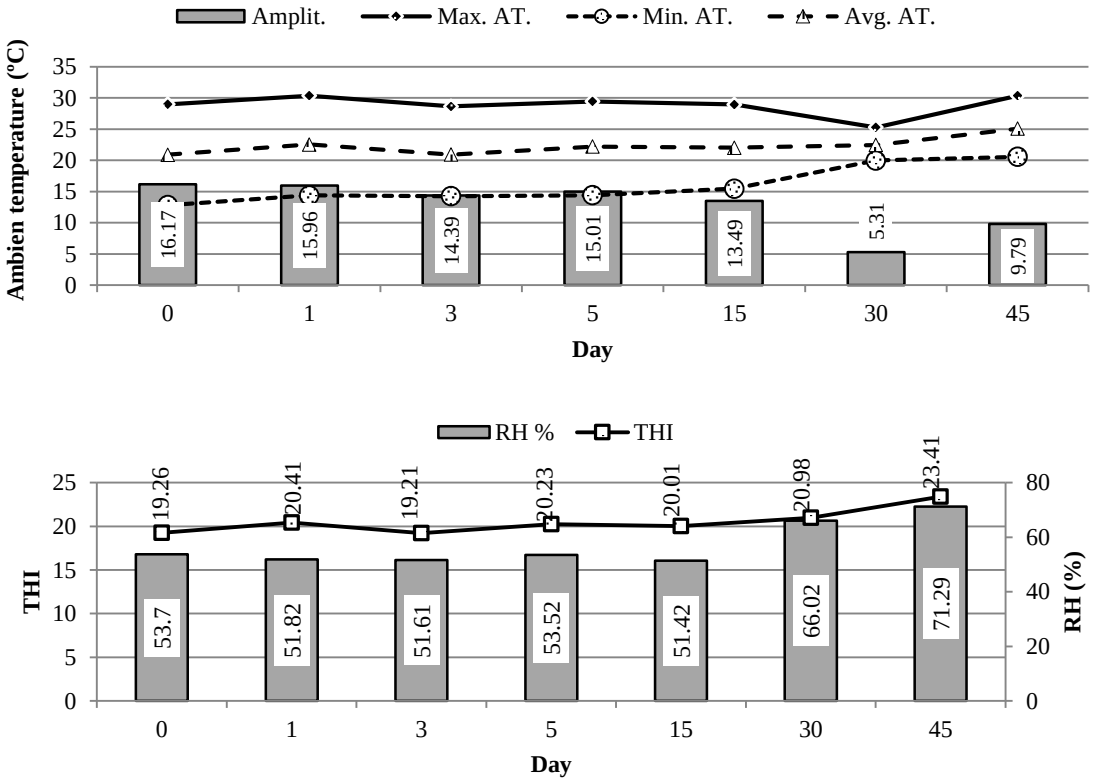

Fig.1: Meteorological data throughout the experiment.

3. 2 Live body weight

Initial body weight was homogeneous as the difference between groups was insignificant; the corresponding values were 35.87 ± 1.625 and 35.62 ± 1.625 kg for of control and shorn groups, respectively. Although no significant differences between unshorn and shorn ewes at days 15, 30 and 45, body weight of shorn sheep declined after shearing and retained lower values than unshorn ewes till day 45 (Fig. 2).

Rather than the subtracted greasy fleece weight in day 0 (1.30 ± 0.141 Kg) which equals to the mean fleece weight, shorn group continued to lose more weight till day 15 were they lost (0.93 ± 0.138kg) of their body weight compared with their body weight at day 0 after shearing. Weight loss declined on days 30 and 45to minor magnitudes (0.45 ± 0.138 and 0.03 ± 0.138 kg, respectively)(Fig.3) to achieve lower but not significantly differentfinal body weight than their initial body weight and the final body weight of the control group (Table 1).Therefore, Mean body weight of shorn ewes (34.15 ± 0.700 kg) was lower (P<0.05) than that of unshorn ewes (36.25 0.700 kg) as they experienced greater body weight fluctuations after fleece removal as shown in Fig. (3). On the other hand, body weight of unshorn ewes increased gradually to end the experiment at higher (P>0.05) final body weight than its own initial body weight.

The analysis of variance showed significant (P<0.01) effect of treatment, day and treatment × day interaction on body weight change that lead to the obvious variations between shorn and unshorn ewes in their body weight responses to shearing and the climatic conditions records at each day point. Fleece provides protection against extreme heat exchange between the naked skin and the ambient air to maintain constant body temperature and lower energy

requirements for thermoregulation [4], [10] and [20]. Therefore, removing the fleece enhances heat dissipation from the body surface to the surrounding environment and shifts the thermoregulatory set point that leading to higher utilization of body reserves to meet the elevated energy requirements[2], [21] and [8]. Regarding the constant nutritional level along the trail, it is logic to expect the body weight loss in shorn ewes. Coincidently, [6] revealed that shorn sheep require more feed intake to maintain their body weight and to compensate the utilized amounts of their body reserves in thermoregulation. Similar results were reported by [27] who revealed that despite the insignificant effect of shearing on body weight of shorn ewes, they still in need to enhanced nutritional levels to maintain their body weight after shearing.

The decreased body weight loss after day 15 indicates the capacity of shorn Barki ewes to regain their body weights. Previous reports referred body weight regain after shearing to some positive effects of shearing i.e. motivating feed intake [24], elevating feed conversion rates [19] and improving the performance of shorn ewe's [17].However, the stimulatory effect of shearing on body weight gain may presumably occur when the stimulating effect on the appetite is greater than that required to meet the increased demand for heat production [2].Moreover, body weight regain of shorn ewes at days 30 and 45 was associated with higher average AT and lower AT amplitude when they experienced a period of more warmth climate for longer day time (Fig. 1). In accordance, no negative effect of shearing was detected on body weight of heat stressed fat tail Awassi lamsas they did not need to utilize their tissue's energy to produce more metabolic heat [10].

Table 1: Least squares means ± standard errors of initial body weight (IBW), final body weight (FBW), mean body weight (MBW) and body weight change rate (BWC) in kilograms as affected by shearing.

Treatment	IBW	FBW	MBW	BW Change
Unshorn	35.87 ± 1.618	36.75 ± 1.634	36.25 ± 0.706 [a]	0.87 ± 1.121 [a]
Shorn	35.62 ± 1.618	34.29 ± 1.634	34.15± 0.706 [b]	-1.33 ± 1.121 [b]

Means with different superscripts in the same column differ significantly (P<0.05).

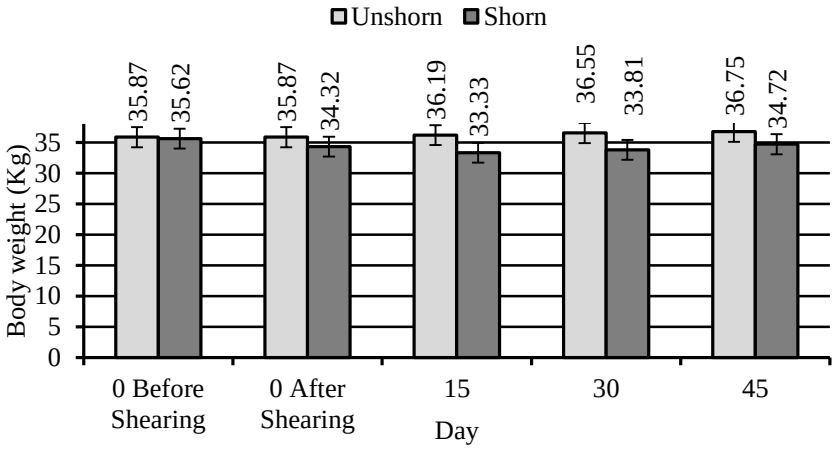

Fig. 2: Body weights of the experimental groups throughout the experiment.

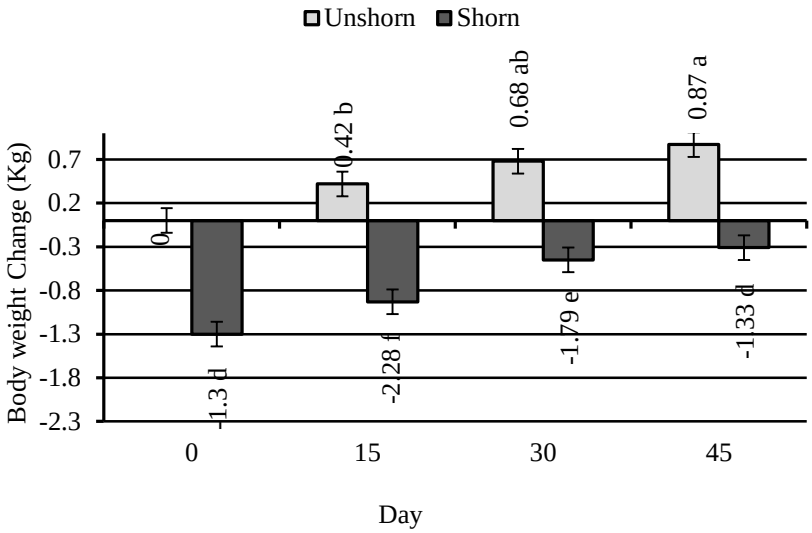

Fig.3: Body weight changes of the experimental groups throughout the experiment.

3. 3 Physiological responses

On the day before shearing (day 0), no significant differences in rectal temperature, skin temperature and respiration rate were detected between unshorn and shorn ewes, confirming the homogeneity of the sheep population. After shearing, shorn ewes recorded lower values of RT ($P<0.01$), ST ($P>0.05$) and RR ($P<0.01$) than their unshorn mates.The effects of time, day and all interactions were highly significant on the studied physiological traits.

Shorn ewes had lower ($P<0.05$) RT than unshorn ones at both monitoring times (39.42 ± 0.779 and 39.80 ± 0.784 °C, respectively). In addition, It also recorded lower ($P<0.05$) RT at 8:00 am (39.25 ± 0.110 °C) than at 2:00 pm (39.85 ± 0.106 °C)(Table 3).

On day1, shearing declined ($P<0.05$) RT of shorn ewes to lower value than that of unshorn ewes at both time points. The corresponding values were 39.66 vs. 39.75 °Cat 8:00 am and 39.12 vs. 40.55 °C at2:00 pm.Moreover, the recorded RT values of shorn sheep were lower ($P<0.05$) than the initial values recorded in Day 0. From day 3 upward to the end of the trail, shorn ewes retained lower RT degrees than the unshorn ones at both time points. RT presents the net result of heat production by the animal and heat exchange to the environment [16]. Therefore the results may emphasis lower metabolic activity in shorn than in unshorn ewes at both time points. [21] reportedsudden temporal reduction in RT that occurs after shearing. They considered this reductionas an effective stimulator to readjust the homoeothermic set point to new level that meet the induced changes in metabolic requirements. Consequently, shearing modifies nervous control mechanisms that collaborate in readjusting the thermoregulatory responses [4]. Physically,bylowering its metabolic rate shorn ewes succeeded indecreasing its core to peripheral thermal gradient toimpede heat dissipation from the body tissues to the periphery andminimizes heat dissipation to surrounding environment [3].

Shearing declined ST at morning time when shorn ewes had lower ($P<0.05$) ST (36.08 ± 0.151 °C) than unshorn ewes (36.69 ± 0.151 °C) while the difference between groups was insignificant at afternoon time. This may explain the insignificant between groups difference in overall ST. Shearing effect on ST extended to longer time compared with the effect on RT as it continued up to day 3though it was limited in morning time where shorn ewes had lower ($P<0.05$) ST than unshorn mates at 8:00 pm.The corresponding values were 36.11 vs. 36.82 °C on day 1 and 35.45 vs. 36.33 °Con day 3(Fig. 4).Shearing in mild and cold climates dramatically reduce body temperature due to the inefficient peripheral insulation of shorn sheep [2]. Lower ST stimulates the subcutaneous cold receptors to modify the metabolic level by decline ST [12]. In addition, changes in ST reflect a complex interaction of several factors that affect the metabolism [15].

Respiration frequency (Breath/minute) was slower ($P<0.05$)in shorn (38.96 ± 0.394) than in unshorn sheep (40.55 ± 0.412). Concerning the effect of time, the difference between shorn and unshorn sheep was significant at 8:00 am where RR was lower ($P<0.05$) in in shorn (37.67 ± 0.545) than that of unshorn sheep (39.59 ± 0.569). On the hand the difference between groups was insignificant at 2:00 pm. This may indicate more sensitivity of shorn ewes to the colder time of the day as they reduced

their RR to minimize heat loss via respiratory evaporation.Coincidently, [26] reported a significant decrease in RR of shorn sheep at morning compared with afternoon time. According to [7], RR reduction in sheep is a thermoregulatory adjustment to the environmental temperature directed at heat conservation. Therefore, shorn ewes reduced theirRR at morning time toreducebody heat loss via respiratory evaporation during the cold time of the day. In contrast, both groupsincreased RR at afternoon to facilitate heat dissipationby increasing respiratory evaporation[9] and [4].

Theresults were in accordance with those of[22] on ewes, [26] on rams and [19] on lambs.According to [18], pulmonary ventilation control aims to balance metabolic needs with homoeothermic requirements.

On day 30, there were observed elevations in RT, ST and RR of both groups that coincided with the lower amplitude between maximum and minimum ambient temperature on this day (Fig 4). This may indicate to effect of exposure to longer time of higher ambient temperature during the day. Generally, the daily RT, ST and RRvalues at both time points coincided with the changes inrecorded ambient temperature and the amplitude between the maximum and minimum temperatures toward the end of the experiment (Fig.1). In accordance,[4],[19] and [26] indicated that shearing alters the circadian and diurnal rhythmsofRT, ST and RR as adaptive responses to modifying the metabolic activity of the shorn sheep to accommodate the shifted homoeothermic set point after shearing. Reducing the metabolic activity leads to conserve body reserves of being utilizedin generating extra heat that could be easily dissipate after fleece removal[5]and may end to body weight loss [6].

In coincidence with [14], by lowering RT and ST shorn ewes succeeded in maintaining their peripheral temperature near to the ambient temperature degrees. Consequently, differences in (RT-ST) and (ST-AT) gradients between unshorn and shorn ewes were insignificant (Table 3). On day1 afternoon, shorn ewes recorded lower ($P<0.05$) RT-ST gradient than unshorn ones due to the obvious reduction in ST recorded on this day at 2:00 pm (Fig. 4 & 5). Amplitude of RT-ST gradient at 8:00 am was about the double of that pointed at 2:00 pm (Table 3). It means that shorn ewes were largely exposed to body heat loss during the cold night and early morning hours especially if (ST-AT) gradient was taken in account. At 8:00 am (ST-AT) gradient was about two folds of its 2:00 pm value. However, the differences between shorn and unshorn ewes were insignificant at any of the monitoring days. This may emphasis that the decline in ST which took place after shearing was an advantageous to the shorn ewes in order to reduce body heat loss to the cooler surrounding environment. At 2:00 pm, when the ambient temperature elevates, the differences between shorn and unshorn ewes in RT-ST gradient were less announced compared with those recorded at 8:00 am (Fig. 5). This may be due to larger amount of body heat loss via respiratory ventilation in shorn ewes at 2:00 pm as mentioned previously (Fig. 4).

Table 2: Least squares means ± standard errors of rectal temperature (RT), skin temperature (ST) and respiration rate (RR) as affected by shearing and time points.

		8:00 am	2:00 pm	Overall
RT (°C)	Unshorn	39.62 ± 0.107 ^{b}	39.99 ± 0.111 ^{a}	39.80 ±0.784 ^{A}
	Shorn	39.25 ± 0.110 ^{c}	39.58 ± 0.106 ^{b}	39.42 ± 0.779 ^{B}
	Overall	39.44 ± 0.078 ^{b}	38.78 ± 0.078 ^{a}	
ST (°C)	Unshorn	36.69 ± 0.147 ^{b}	38.42 ± 0.152 ^{a}	37.52 ± 0.133
	Shorn	36.08 ± 0.151 ^{c}	38.40 ± 0.146 ^{a}	37.28 ± 0.132
	Overall	36.39 ± 0.106 ^{b}	38.41 ± 0.106 ^{a}	
RR (Breath/minute)	Unshorn	39.59 ± 0.569 ^{c}	41.51 ± 0.569 ^{a}	40.55 ± 0.412 ^{A}
	Shorn	37.67 ± 0.545 ^{b}	40.25 ± 0.545 ^{ab}	38.96 ± 0.394 ^{B}
	Overall	38.59 ±0.398 ^{b}	40.85 ± 0.398 ^{a}	

Means with different lowercase superscripts in the same row and uppercase superscripts in the same column differ significantly ($P<0.05$).

Depending on the changes in physiological responses induced by shearing, it could be concluded that shorn Barki ewes may have the capacity to regulate their metabolic activities when experience mild cold exposure by heat retention mechanisms rather than substantial increased heat production to lower body weight reduction due to utilization of their energy reserves. Therefore, it may be of importance to retard shearing to period of more warmth climate to avoid any negative effect on the body weight of the sheep herds particularly under limited levels of nutrition.

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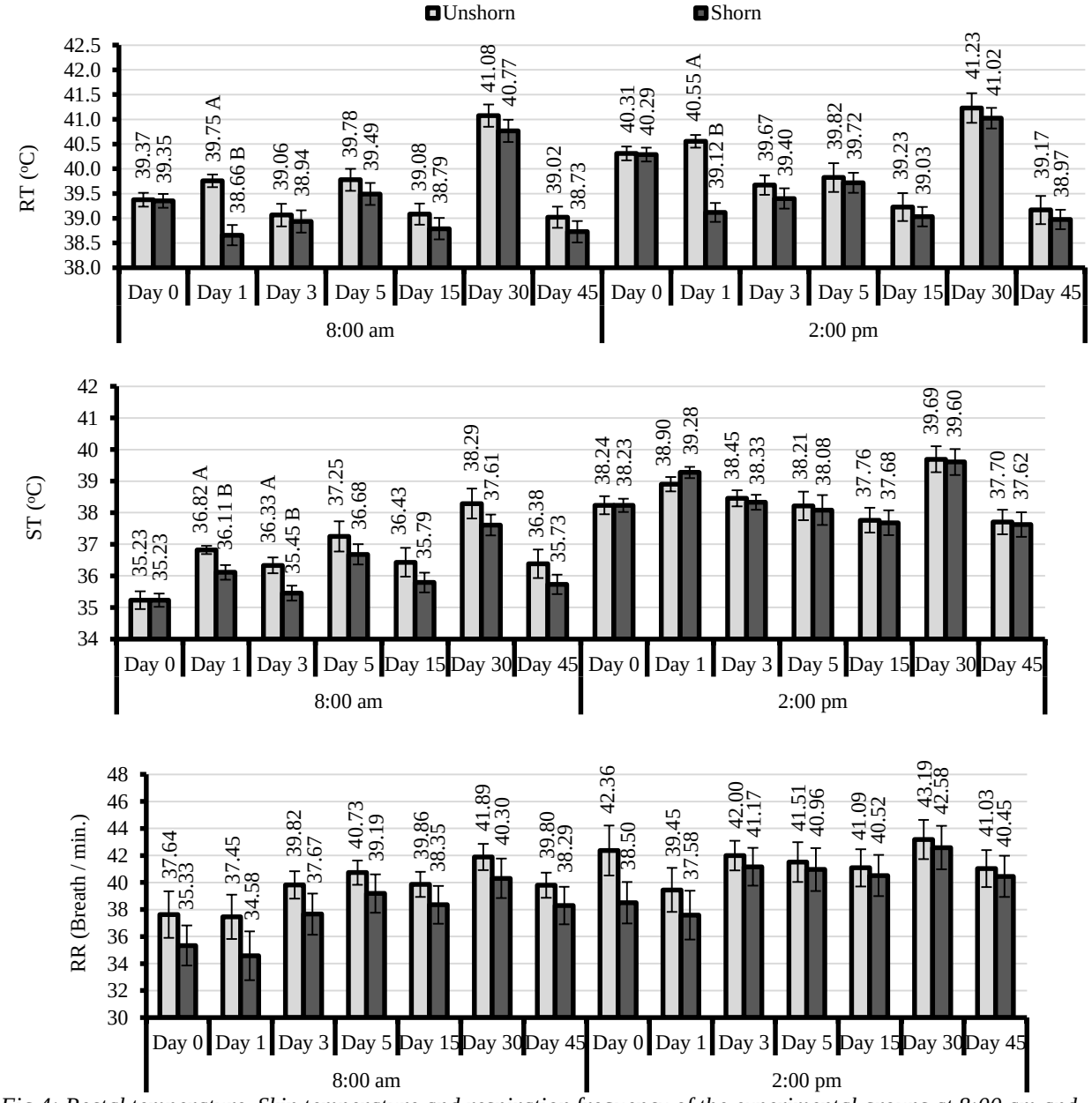

Fig.4: Rectal temperature, Skin temperature and respiration frequency of the experimental groups at 8:00 am and 2:00 pm time points throughout the experiment.

Table 3: Least squares means ± standard errors of (RT-ST) and (ST-AT) thermal gradients as affected by shearing and time points.

		8:00 am	2:00 pm	Overall
RT-ST (°C)	Unshorn	2.92 ± 0.156 [a]	1.57 ± 0.162 [b]	2.27 ± 0.131
	Shorn	3.17 ± 0.161 [a]	1.17 ± 0.155 [b]	2.14 ± 0.130
	Overall	3.04 ± 0.112 [a]	1.36 ± 0.112 [b]	
ST-AT (°C)	Unshorn	19.99 ± 0.502[a]	9.57 ± 0.521[b]	14.97 ± 0.531
	Shorn	18.99 ± 0.518[a]	9.75 ± 0.499[b]	14.20 ± 0.527
	Overall	19.50 ± 0.360 [a]	9.66 ± 0.306 [b]	

Means with different lowercase superscripts in the same row differ significantly (P<0.05).

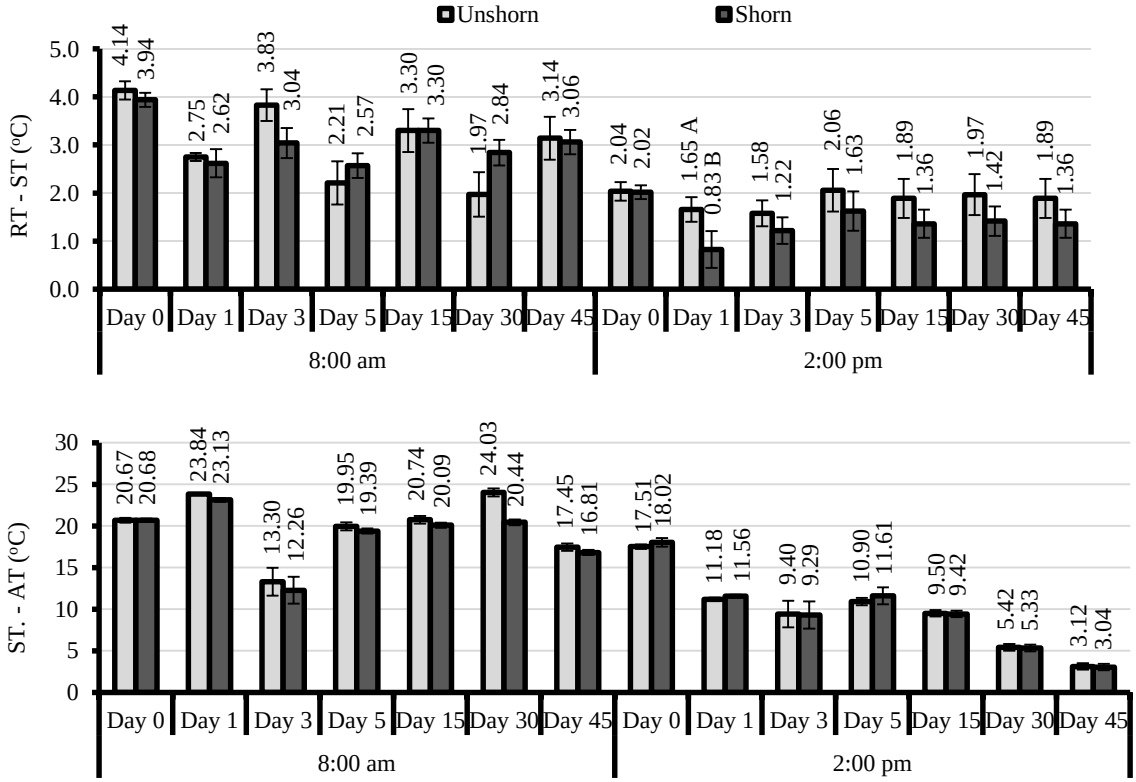

Fig.5: (RT-ST) and (ST-AT) thermal gradients of the experimental groups at 8:00 am and 2:00 pm time points throughout the experiment.

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Climate Change Adaptation Planning, Measures and Multilevel Governance Approaches in Pakistan: Climate change and its risks on natural resources and human health of the country and Governments' responses

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***Abstract*—** Climate Change is a *complex challenge that the world is facing. Pakistan would be the most affected by climate-related disasters because of a lack of proper institutional, financial, and technological mechanisms to control the risks of climate change (Yamin, et al.,2005). Past natural disasters show that the local governments with insufficient financial resources, planning, institutional, technical adaptation capabilities remain unable to overcome natural disasters.*

Pakistan is an agricultural country, and its economy relies on the agriculture field. However, the growth rate of agriculture is decreasing because of environmental changes that are having great harmful effects on the already limited water resources(The World Bank Group, 2016; Khan, et al., 2016). Long-term climate change and frequent extreme climate events have significant negative impacts on people's health that can cause death, disability, or suffering worldwide, especially in developing countries.

This paper will recommend climate change adaptation and measures to reduce environmental degradation *that will help create practical results for disaster reduction, public health, and water and natural resources' improvement of Pakistan.*

Keywords*— *Climate Change Adaptation, Disaster Reduction, Human Health, Natural Resources Improvement Polices.

I. INTRODUCTION

In the twenty-first century, climate change is a severe risk for the economy, resources, and security of the world. Its adverse effects are being felt all over the globe. Climate-related disasters have affected millions of people on our planet. They are expected to affect millions of people, especially in developing countries, because their economy and social development heavily rely on climate-sensitive natural resources. Along with this, developing countries have less skill to control the impacts of environmental degradation effects. (Yamin et al., 2005).

It is expected that adverse health impacts of weather and climate will be on people of all ages, especially the greatest on older people, children, subsistence agriculturalists, and indigenous or tribal people in low-income countries. People's health and healthcare management systems have now improved worldwide as compared to the past. However, healthcare improvement, population health, and care management arrangements are not the same in every country in the world. The average years of life have increased in some parts of the world over recent decades. However, life expectancy has already declined in many countries of the world, especially in developing countries during the last twenty years due to the spread of epidemic diseases such as HIV/AIDS, Ebola virus disease, and coronavirus. Extreme climate events can rise further the vulnerability of human health because climate-related extreme disasters force people to migrate to other unsafe places. As a result of forced displacement due

to climate-related disasters, people lose the stability of their families, and they are more likely to be infected by epidemic diseases (IOM, 2009; IPCC-WGII, 2007).

Pakistan's climate is generally warm and lies in the world where the temperature is rising even faster than the global average, rainfall patterns are changing rapidly, and the sea level is rising due to ice melting. All these changes contribute to extreme events in the form of heat waves, earthquakes, tornadoes, flooding, and droughts(Rasul & Ahmad, 2012). Pakistan is prone to a variety of climate-related disasters. These disasters have made history in Pakistan because of their harmful effects on the residents' health, on the natural resources, and the economic health of the country. The issue is that climate-related natural disasters, whether they are extreme or small scale, will continue to occur. Most of the natural disasters happen suddenly again and again with little or no warning and have many side effects at the national and the local levels(Wei Choo, 2008).

Pakistan is mostly an arid and semi-arid country, with approximately 80% of its area falling in these categories. Since independence in 1947, many natural resources of Pakistan have been damaged, and a large portion is under significant risk every year due to the natural disasters, the lack of planning, awareness, and control before, during and after the disaster events (Iqbal, et al., 2014). The negative consequences of natural disasters are being seen in different locations with severe effects on the food production, human health, the infrastructure, the resources and the livelihoods of Pakistan (United Nations Environment Programme (UNEP), et at., 2013).

In general, when any climate-related natural disaster occurs, people look towards local governments and related agencies for immediate relief aid and transitional recovery assistance. Over the past decade, Pakistan has faced many natural disasters. Unfortunately, the past natural disasters show that the local and the central government departments and the related national agencies could not provide immediate relief and recovery to the affected people during an emergency(Mayo, et al., 2013). The core cause for the low performance of the concerned departments is that they have a lack of adequate response mechanisms and coordination platform among the line agencies, the lack of skills of the local officials and the lack of strategies for dealing with the natural disasters (Ainuddin, et al., 2013).

Local governments can better react to the risks of environmental changes. They can produce positive results in terms of reduction of local pollution, collection waste, natural resources management, and economic growth in a situation where local data and funding for adoption are available, and adaptive responsibilities are clear (National Climate Change Adaptation Research Facility, 2012). Pakistan is on the front line of the climate crisis and its adverse effects. There are a lot of obstacles to coping with environmental degradation. Still, the common obstacles within the local governments' context in the world can be a result of societal and organizational behavior, inconsistent governance structure, financial, economic and regulatory obstacles, and insufficient information, awareness, and knowledge of environmental problems (Jensen, et al.,2016). Adaptation and the adaptive capacity building to deal successfully with gradual changes to climate is not a one-time process. On the contrary, it is an ongoing practice of training, response, and correction(The World Bank Group, 2011). Building effective adaptation strategies to control environmental degradation that is operative from the local to the national government level will support the country's economy.

Devolution of powers from top to bottom and effective and decentralized strategies to control climate-related national disasters can significantly reduce the loss of lives, the loss of infrastructure, and properties that happen due to environmental degradation (Mayo, et al., 2013). The climate change decision making and planning process is in the hands of politicians of a country. Climate change can only be tackled effectively if the policymakers increase the ability, involvement, resilience, and skill of the local public and involve both the local officials and local non-profit organizations in the climate change actions and policymaking process (Ainuddin et al., 2013).

In light of the empirical evidence, this paper will examine the environmental degradation awareness, its impacts, and the adaptation system and process in Pakistan. It will argue that climate change effective policies, planning, and activities at the local government levels can produce positive effects for disaster reduction, natural resources and human health improvement, environmental protection, and economic and community development in Pakistan. The paper will identify those challenges and provide analysis about the main obstacles to adaptation and what adaptation practices to control environmental degradation are planned to overcome climate change impacts. Further, the possible role of Geographical Information System (GIS) along with the idea of environmental awareness, will also be evaluated for improving awareness about environmental degradation. Finally, this paper will identify and recommend some specific strategies, realistic policies, and practical solutions

for developing and strengthening the capability of the local governments.

Objectives

- To examine the Pakistan government's response to climatic change and impacts of climate-related events on Pakistan's natural resources, and people's health
- To evaluate the awareness level of environmental degradation and its effects among local government employees
- To review existing climate change policies and the adaptation systems, and planning and assess the adaptive capacity of key stakeholders
- To identify significant gaps in climate policy and planning and how it can be filled in a successful manner

Research Questions

- How Pakistan's serious problem of limited natural resources and low environmental awareness will change climate change consequences for the country?
- What are the main obstacles to adaptation, and which adaptation policies are already planned to reduce climate-related disasters at the local levels in Pakistan?
- Which adaptation system, policy, and practice would be the best in Pakistan in reducing climate change adaptation barriers?
- Will the decentralization of government disaster institutions be crucial to reducing the disaster impacts at all levels?

II. STUDY METHODS

Several methodologies will be used to gather information in writing this paper. These include literature studies and secondary sources, case studies and research papers, surveys, and evaluation reports of climate change challenges and adaptation areas. The paper is an examination and analysis of journal articles, dissertations, research analysis, and books from different sources. These documents and reports were obtained from the Ministry of Environment (MoE); the National Disaster Management Authority Pakistan (NDMA); the Ministry of Climate Change(MCC): the Ministry of Finance; the Provincial Disaster Management Authority (PDMA); the Indus River System Authority; the Pakistan Flood Commission; and the Pakistan Agriculture Research Council, World Health Organization (WHO) and Pakistan Planning Commission. Besides these sources, the paper will use information from the reports, documents, and policy briefs obtained from different semi-governmental and non-governmental organizations working in and for Pakistan.

Also, the paper will use the developmental working knowledge gained at the national and international levels in the project management, public healthcare, and emergency preparedness field. Furthermore, The learning experience from international professional training courses and conferences on the subject of environmental management, disaster relief, and public healthcare is also an asset that has been very helpful in identifying major obstacles to the adaptation of climate and natural resources management policies and strategies. Based on a literature study and our working experience, the paper will recommend various possible practical solutions for the Pakistan government that would strengthen the local governments and empower the communities to combat environmental change.

III. LITERATURE REVIEW

The literature review section will provide an overview of climate change and its impacts on natural resources and public health, identify priority concerns, and focus on environmental change adaptation measures, mitigation, future challenges, and find the role of climate awareness and the local government in developing adaptation. Finally, based on the relevant literature, the paper will discuss the climate change response strategies of different countries and find the most vital sectors for environmental change adaptation.

What is Climate Change

Environmental change and degradation, which is a long time variation in the world's temperature or seasonal patterns, affects people, agriculture, sea levels, and ecosystems and increases the risk of natural disasters (UNEP, 2013). The climate is changing due to the use of fossil-fuel burning, the release of industrial waste, the burnings of agricultural wastes, the use of insecticides and pesticides, mining, and deforestation (Simeone, 2006). These human activities increase the level of greenhouse gasses (GHG) / carbon dioxide (CO2)in the air and produce greenhouse effects. According to the United Nations Framework Convention on Climate Change (UNFCCC), human activities are believed to be changing the structure of the earth's atmosphere. Environmental change is happening primarily due to a rise in the amounts of aerosols (small particles), cloudiness, and greenhouse gasses such as carbon dioxide (CO2) (UNFCC, 1992).

Climate Change and Public Health

Air pollution and global warming can significantly influence human health and increase disease mortality. Non-infectious diseases such as cancer, kidney disease, depression, and heart disease are multiplying in developing countries. Because environmental change has a strong relationship with public health that has caused and will create more enormous implications for human health. According to Baaghideh 2017, rising global temperatures and air pollution will increase the mortality rate of non-infectious diseases in future decades.

Climate Change Adaptation plus Mitigation

The common meaning of the word "adapt" is to make and become suitable for something or follow an idea, method, or course of action. According to the UNDP, the word adaptation means "all changes in a system, compared to a reference case that reduces the adverse effects of climate change" (United Nations Development Programme, 2003). The IPCC says that "adaptation is a modification in environmental, societal, or financial structures in response to sincere or anticipated climatic variation and their reactions or impacts" (IPCC, 2016). All the definitions give the idea of regulation, climate variability, and extreme natural disasters' reduction rule. All the above explanations of adaptation of natural resources and climate management express that it is a modification, improvement, and arrangement in institutions and in structures to mitigate the expected impacts of environmental change and climate-related disasters. Simply climate change adaptation means that taking an effective action to lessen the extreme effects of environmental-related disasters in the most effective way by taking benefits of opportunities that can occur anytime, for instance, developing an effective early warning system, using limited natural resources more efficiently, building higher and stronger river walls and dams to prevent floodwaters and switching to more drought-tolerant crops, etc.

Mitigation refers to any strategy or act that has been taken to lower the severity of environmental change impacts in the long term. According to the UNEP, climate change mitigation is the action and strategy of using new or old technologies, equipment, or management practices that lessen the greenhouse gasses, which are released into the air (UNEP Climate Change Mitigation, 2016).

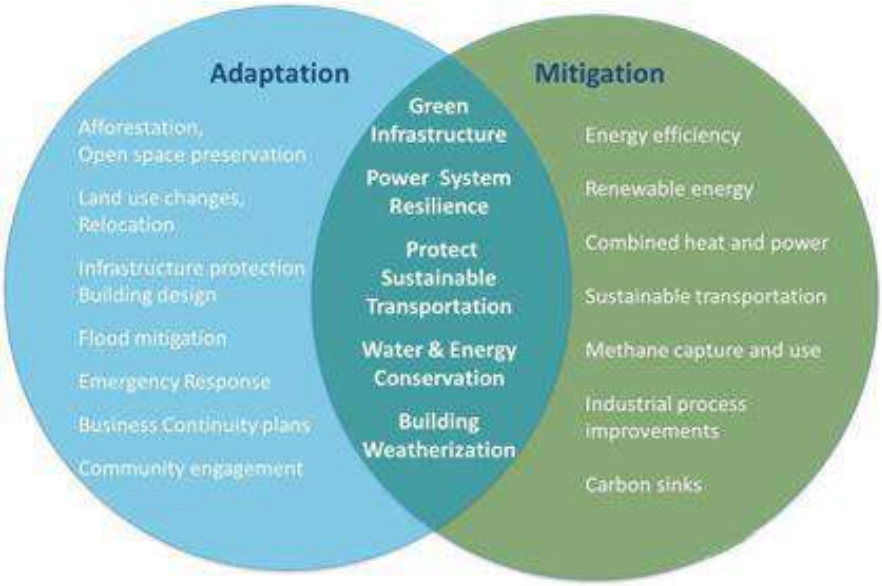


Fig. 1. Adaptation Plus Mitigation Synergies

Source: (The Center for Clean Air Policy, 2013)

Adaptation and mitigation strategies to climate degradation are both compulsories to decrease the dangerous outcomes of environmental change because adaptation in the field of environmental degradation refers to the capability of a system to mitigate the harmful outcomes of environmental change and enhance the resilience of disaster-prone country and people.

Local Governments and Climate Change

The Stern and International Energy Agency (IEA)'s report says that metropolises are known as the largest producers of GHGs emissions; they are responsible for up to 75 % of the global emission of GHG. So, researchers of the cities' governance on climate degradation have grown up rapidly during the last ten

years, and cities' responses to environmental change have grown all over the world. For more than a decade, at an international level, there have been many debates and discussions between nation-states to reduce the emission of GHGs. Still, it has been seen in an international relations practice that global environmental governance often takes place at the global level. However, many reports suggest that the nation-states will not be able to meet their international commitments for dealing with climate change without precise engagement with subnational and local governments' action plans. Carbon emission originates from the actions that are rooted in cities, so the local government is the most appropriate domestic authority to address the specific global environmental problem and for bringing a depletion in CO2 (M.Betsill & Bulkeley, 2006).

The International Council for Local Environmental Initiatives (ICLEI), one of the leading institutions of local governments were formed first in the 1990s by the U.S and Toronto federal government and numerous private organizations to address the climate change and environmental issues of local governments internationally. In 1990, The second institution, "Climate Alliance," was established as a union between European metropolises and the public to protect the rainforest and reduce 50 percent GHG emissions by 2030 (Bulkeley, 2010). The Cities for Climate Protection was third initiated with the support of the European Union (EU)in 1993 by the local governments of the UK, France, and Germany. This initiative was taken to give the authority to local governments to protect the climate and the environment at local levels through measurement, obligation, planning, executing, and monitoring(UN HABITAT & ICLEI, 2004). Through these international networks, the substantial responsibility of the local government in environmental change governance is acknowledged as crucial to the advancement of a country. Currently, the agreement of international networks maintains the local governments' importance and recognizes that local governments convey the local needs more clearly and quickly than the national levels of government. According to the) and these three transnational municipal networks, a local government has a great experience dealing with environmental issues and more realistic compared to higher government levels, and local government has the authority to overcome any circumstances in a more sustainable way. The local governments of many countries are joining these networks, and the members of these networks are increasing every year. But despite the international policy interventions at the local government level since 1990, there is an enormous gap so far in the implementation of actual actions to reduce GHG emissions. Consequently, one significant benefit has achieved during the last several years that the debate on international climate policy has been shifted to the agenda of local politics (M.Betsill & Bulkeley, 2006).

Challenges to Climate Change Adaptation

The scale and severity of natural hazards, for instance, severe thunderstorms, floods, earthquakes, glacial melting, and droughts, pose severe threats to all sectors of society. They have grave impacts on the development, health sector, and ecosystems of a country. Although climate change is a severe problem, governments usually give less importance to it than other national issues (Lorenzoni & Pidgeon, 2006).

Lorenzoni and Pidgeon (2006) and Neuhoff (2015) indicate in their books that nowadays, policy indicators have gained an increasing level of attention in developed and developing countries. There can be many obstacles that can hinder the implementation of policies. These books offer some significant suggestions, or obstacles as barriers to climate adaptation, which are:

Effective Adaptation…

(1) needs coordination, awareness, and training
(2) wants more capital
(3) needs professional skill
(4) needs to add with regulatory requirements
(5) is a complicated process and others will take benefits

Climate Change Adaptation Measures

Both policies and measures are required for climate adaptation in different ways. Policy refers to decisions, instruments, and plans with the means of implementation to change economic structures and individual behaviors. For instance, a water supply policy of a county may include many instruments and policies, which may include dam construction plans, rainwater harvesting, and artificial wetlands plans, etc. In contrast, measures refer to a course of action that is taken to achieve a specific purpose. In a climate change adaptation context, measures are actions that prepare a country for the consequences of environmental change and work according to the chosen policy. Environmental change adaptation measures contribute to the national goal. They are designed for both current and future consequences of environmental change, such as the implementation of water supply and public health programs, extreme events prevention projects (OECD, 2006; Burton et al., 2005).

The following options are essential adaptation measures to prevent, reduce, and mitigate short to long-term effects of environmental change.

- Adaptation Measures
- Policy Measures
- Economic Measures
- Technological and information systems Measures
- Capacity building for staffs
- Monitoring and Predicting
- Risk Sharing and
- Awareness Raising

Climate change adaptation measures are usually specific actions that prepare a county and communities to decrease vulnerability to the consequences of environmental variation(San Diego Forward, 2014).

Environmental Literacy

Folks' environmental awareness and education are vital, which is the most potent tool for climate change awareness and prevention. The primary purpose of climate awareness is a behavioral change of people so they can act in a more environmentally friendly way (UNEP, 2007). Environmental knowledge and education play an enormous part in raising awareness of environmental challenges and shaping the attitudes and behaviors that can protect the environment, human health, and natural resources of a country and contribute to the sustainable development of a country (The British Council, 2013). A lack of education and awareness on environmental issues make a country more grievous to the dangerous effects of environmental change. Generally, people do not have much environmental awareness, and they do not know how to conserve the climate and use natural resources in an environmentally sound manner (Srivastava, 2016).

Dissemination of environmental knowledge, skills for mitigation and adaptation at a country level are crucial for environmental protection and improvement of the health of the environment and people. It must be an essential part of the education system at the national level. Environmental training and awareness give not only knowledge to people about environmental change, but also, it develops thinking, and implementation skills of environmental protection and this necessary expertise can be conveyed at the grass-root level by including in the curriculum of schools. According to United Nations Educational, Scientific, and Cultural Organization, there are two main strategies for the development of environmental change awareness and skills on a country level among citizens and professionals that contribute to the solution of the environmental problems(Srivastava, 2016; UNESCO, 1992).

1) By creating climate change awareness of the public through the education system.
2) By providing environmental education and practical training through professional groups.

Why Local Government?

Local government is an administrative body at the local level that delivers services and goods for small geographic areas. It is divided into three major types: rural government, urban government, and the provincial government (UNDP et al., 2010). Local government is an ideal place to manage environmental change and its devastating effects on natural resources and folks, but in many countries, adapting to environmental change is a new topic, so no detailed reports and researchers are generally available at local government level regarding climate change adaptation and measures. There is no one easy, short, or magic formula to tackle climate change. Some countries are focusing on actions to reduce GHGs at city levels, and some are working to mitigate anticipated adverse environmental impacts at the local levels(National Research Council, 2011). These countries are trying to manage the negative impacts of the environment and reducing the magnitude of global warming. Most of the climate change discussion on the discipline of environmental variation and its strategic implications have been shifted at an international level rather than the local or national level. As a result, local governments and officials are not well familiar with the knowledge of the environment and environmental degradation and its strategic implications. They do not contribute effectively to climate change mitigation (UNDP et al., 2010).

Local government can play important roles to increase community adaptation capacities and to take leadership responsibility and actions in setting the environmental change policies, standards for environmental protection, for the protection of human health and pollution control. Because the local governments directly include all local public-sector development programs and they have good knowledge of the delivery of government services, and local government officials are the first responders when climate-related disasters occur(Morrill, 2016). Therefore, local governments can achieve a higher success rate in achieving the objectives of environmental, natural resources, and

human health protection (The U.S. Department of State publishes eJournal USA, 2011).

Conclusion

The study has revealed through the literature review that variability of the environment has many implications from the household to the national and international level because it is not just an environmental matter. Climate impacts will be most significant and will have huge impacts on every sector of the global economy. Effective adaptation of natural resources and environmental management, climate change literacy, and awareness, environmental change mitigation, and actions are vital to mitigate the likely destructive effects of climate degradation on people's health, natural resources, and the environment. Long-term changes in the environment will create more extreme weather disasters that will put severe pressure on people, the local governments and its institutions, natural resources, and the environment. It will be necessary to find the best practices, policies, and tactics to tackle environmental change and its negative impacts.

Discussion

The discussion section will include information about the harmful effects of environmental change in Pakistan, strategies to address it, and the deficiencies in those strategies.

The Country Status

Pakistan is highly vulnerable to extreme weather disasters due to its topographical position, high population density, weak institutions' planning, and the economic condition of the country. According to the German Watch Report 2013, Pakistan was ranked as 8th in the countries that were most affected by climate change events (Germanwatch, 2013). Most parts of the country's land are dry, parched, and barren, with very few forests due to the variabilities of natural atmospheric processes. Thus, droughts occur mostly in southern parts of the country, which kill animals and crops and leave people with a low supply of food. The natural environment of the country is severe, and the arid parts have fluctuating temperatures, e.g., in the cold season, it decreases, and in the hot season, it rises. The northern parts of Pakistan are considered dry areas and have little rainfall because, in these areas' monsoon rains do not occur.

Environmental Effects in Pakistan and Local Governments

Climate change and its impacts will not only affect the economic growth, the natural resources, and the people's health of Pakistan, but also it will obstruct the accomplishment of the Sustainable Development Goals (SDGs). The effects of extreme climate hazards on the people and the development of Pakistan can be assessed by the fact that climate-related disasters have killed about 6,209 people and affected nearly 1, 8,521,926 people in the period between 1995–2004. During the period from 2005 to 2014, climate-related disasters have killed about 82,802 people and affected approximately 49,784,339 people (IFRC, 2015).

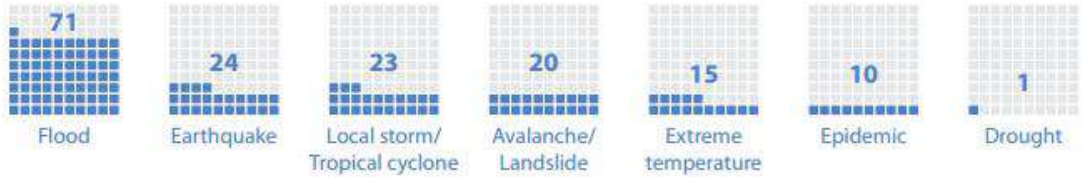


Fig. 2. Number of Climate Disaster Events in Pakistan Since 1900

Source: (UN OCHA, 2011)

Climate change and extreme weather events have already created many problems for Pakistan. Pakistan's provisional governments, including district governments, have been unsuccessful in tackling natural disasters effectively because the central government of Pakistan did not introduce a sound local government system to deal with natural disasters. The central government passed an act in 2013, in which relief efforts, disaster management activities and environment control schemes will be under the control of the local governments which will be responsible for disaster management, providing disaster relief efforts in the event of any disasters and the local governments will prepare and implement environment control schemes. Still, unfortunately, the central government has failed to institutionalize the district's level management authorities so far. The disaster or emergency dealing departments are working in an uncoordinated way to reduce the harmful effects of disasters; for example, the Rescue 1122 Service is one of the largest emergency services in Pakistan providing relief during an emergency, and disastrous situations. But this department is not working under the PDMA. Similarly, irrigation,

agriculture, and health departments are performing their jobs at the provisional levels in an uncoordinated way. They are not performing their jobs according to the National Disaster Management Act, 2010, which was designed to deal with the disasters and the climate (Sharif, 2013).

The Most Vulnerable Sectors to Environmental Change

National Climate Change Policy (NCCP) 2014-2030, UNEP, 2000 and Piracha, et al., 2016 reports have identified the most climate-vulnerable sectors for Pakistan. These include the water, agriculture, and food sectors. Climate change has already harmfully affected the drinking and marine water resources. It will further affect water sectors, which will increase the possibilities of water contamination and create challenges to human health and lead to Water-related Illnesses. Water resources, including rivers, lakes, dams, and underground aquifers for Pakistan, are like tires of a vehicle. So, without tires or with puncture tires, a vehicle cannot run properly.

Similarly, a country with severe water scarcity becomes disabled in terms of development and economic growth. In Pakistan, water protection planning and awareness are minimal, creating a problem for the people, and water resources are under severe strain due to climate change and overpopulation.

A survey was conducted by Piracha, et al., 2016 in local union councils of Lahore, Pakistan, to ask the current and upcoming consequences of environmental degradation in Pakistan from the local officials. The opinion of the local officers shows in the study that environmental variation has already affected the water and food sectors and will continue to affect these resources.

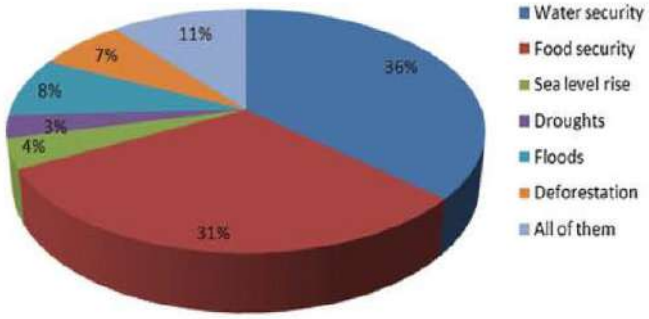


Fig. 3. Local Official's Opinion about the Most Unsafe Sectors to Climate Change.
Source: (Piracha, et al., 2016)

The water crisis will damagingly affect the agriculture sector. Agriculture is the core industry of Pakistan's economy that contributes 25 percent of Pakistan's Gross Domestic Product (GDP) and 60 percent of export earnings, and the industry provides employment opportunity to about 60 percent of the population. Furthermore, both population growth and climate change put pressure on the already insufficient and scarce water resources of the country. It is assessed that the need for water in Pakistan will increase five times from 2020 to 2050 because of population growth. The country has the most extensive canal-based irrigation system, but it is facing critical water shortages due to environmental degradation and poor planning and water management.

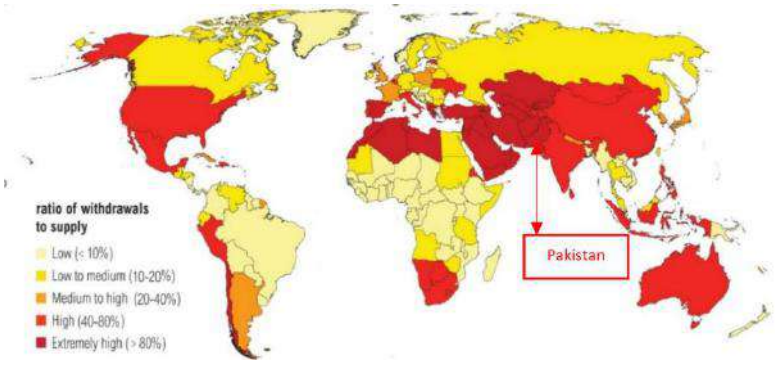


Fig. 4. Most Expected Water-Stressed States by 2040.

Source: (World Resources Institute, 2015)

In the above map, Pakistan is located in an area where many countries of the world will face extremely high-water stress by 2040. There are many studies available in Pakistan on the water crisis, water

scarcity, adverse environmental effects on water resources, and crop yield. However, these studies do not provide details about adaptation strategies in the water sector. One study is available so far that was carried out by the UNEP that provides details about climate change adaptation efforts on Pakistan's water, and the adaptation measures need to be adopted in Pakistan. The study suggests that the water protection, management, and sustainability can be achieved in Pakistan by the construction of small dams, retention pond systems, water conservation measures in rural and urban areas, and through awareness of the people and the farmers regarding water supply issues(UNEP, 2000).

Obstacles to Adaptation in Pakistani Local Governments

The past climate change-related disasters indicate that the government line agencies and the local governments with limited financial resources and lack of technical skills remain unable to control catastrophic natural disasters. Lack of awareness, coordination, and training has always been the main reasons for the low performance of the local government's departments and government line agencies. The Pakistani constitution says that the local governments of Pakistan are a provincial subject (Mayo et al., 2013). They will work under the provincial governments; thus, all Pakistan's four provincial governments will give instructions to their respective local government's legislation (Sansom et al., 2013, p.47). The National Disaster Management Act, 2010 of Pakistan, has categorized three stages of intervention: (1) unions, (2) tehsils, and (3) districts level governments. But unfortunately, the provincial governments have all been unsuccessful in introducingng a genuine and effective local government system so far that have the ability to control extreme environmental degradation and manage frequent disasters at the local levels(Mayo et al., 2013).

Based on the study of three research papers of Janjua (2011), Mayo (2013)and Shahid (2015) which were conducted in Pakistan on the subject of an adaptation strategy and local government, these studies have found and identified that there are three significant obstacles to environmental change adaptation at the local levels in Pakistan.

These obstacles are:

1. The lack of data and adequate awareness
2. The lack of sufficient/effective methods, policies or tools at the local government level
3. The lack of adequate funds

Pakistan and International Protocols on Climate Change

Pakistan is a vast and diverse state with a range of seasons, socio-economics, agricultural patterns, topography, and variable conditions of weather. The country is trying to establish a balance between the economy, people's development, and climate protection. In this regard, as an official unit to many international agreements and conventions, Pakistan has been vigorously involved in the environmental protection meetings and the global climate conferences from its creation in 1947. At the international level, Pakistan has signed and ratified fifteen Multilateral Environmental Agreements (MEAs), conventions, and protocols (MCC,2016). The international commitments of climate help to provide steps to fight against environmental degradation at the country level. Thus, the NCCP is trying to put international environmental change adaptation actions in its national policy frameworks. However, many adaptation strategies at the national level have been unsuccessful that don't include both negative and positive sides of climate change. For instance, the emergency and disaster risk management strategies of Pakistan don't recognize the climate change perspective. It does not cooperate practically with the like-minded local government departments to decrease the vulnerability to environmental change(Khan et al., 2016). Furthermore, the financial resources and capacity to tackle environmental change and its consequences at the local level are not considered in the National Environment Policy (NEP) and NCCP. Nor is there any research available publicly in terms of the local government involvement concerning environmental change adaptation and implementation of the policies.

Environmental Knowledge in Pakistan

Environmental awareness and public education in Pakistan can lead to better environmental planning and management (Awais & Zareen, 2016). According to UNESCO, 2012, educational attainment, from necessary to a higher level, is very low in Pakistan. The country ranks in the second position, where about five million children were out of school in 2012 only. Furthermore, Pakistan has almost 49.5 million illiterate adults, and this is the third-highest position globally. The high rate of literacy in a society can reduce the process of environmental management. According to Howe, 2009, literate persons are more concerned about environmental matters (Howe, 2009).

A detailed survey was carried out in the 150 local union councils of Lahore, Pakistan, both in the urban and rural areas by Awais & Zareen, 2016 to find out the awareness level about environmental degradation among the local government officers. The findings of the study show that 53 percent of the local government officers of

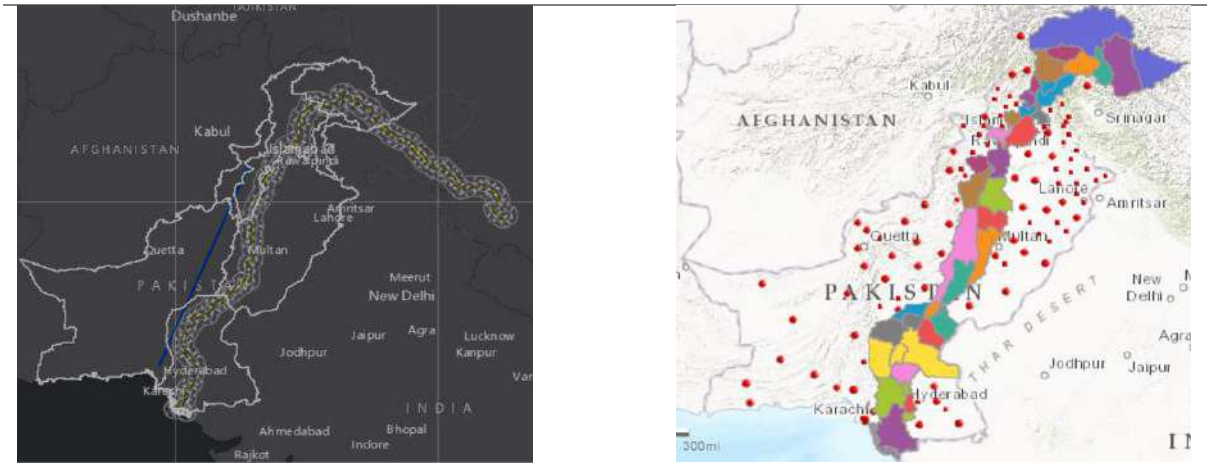
the countryside councils were not aware of the phenomena of environmental degradation, and only 47 percent of local officers of urban union councils had some knowledge of environmental degradation.

GIS's Role and Environment

GIS can be used professionally for developing climate change maps, increasing climate change awareness, and addressing climate-related disaster management processes(ESRI, 2014). Maps and visual displays are a better way of communicating information between the local inhabitants and different public agencies. In this way, they can better understand a complicated climate situation. The latest studies of Awais Piracha (2016) and Zareen Shahid (2012) show that the local government officials in Pakistan do not have the knowledge of GIS and they do not know its importance in climate change planning, managing and mitigating natural disasters (Piracha, et al., 2016; Zareen, 2012). Lack of environmental awareness and lack of knowledge among local people and local government officials can impede climate adaptation in Pakistan.

Floods are very common and cause huge damages to infrastructures, crops, agriculture lands, human lives, and their properties. Pakistan was ranked 5th in those countries in which the highest number of people have affected by floods. Extreme flooding events are considered a reason for forced migration that Extreme flooding events are considered a reason for forced migration. Climate-related forced displacement can increase various factors, for example, it can create social, hygiene, sanitation, and health issues for both the host and migrant, increase pressure on infrastructure and services, weaken the economic growth of the host communities(IOM, 2009).

Pakistan's central government, with the support of the local governments, must develop flood risk GIS maps and mitigation strategies for each district, taking into account the rainfall intensity, the geology, and the land surface factors. These GIS maps and mitigation strategies can be used as a flood awareness and flood decision-making tool. This early warning tool can save the economy, human health and infrastructure of the country, millions of people, and their crops.



The excessive input of water in the Indus River in Pakistan during Pakistan Floods 2010-2011	The districts are selected around the Indus river that might affect the selected areas' crops, people and the infrastructure

Fig. 5. Indus River in Pakistan and Districts Expected to Face Flood Risk.

Source: GIS Map Project, created by Shahzad Ismail

In Pakistan, a few public organizational units have completed some GIS projects to support the disaster management process and to help the NDMA like the NDMA Maps, 2016, the PDMA like PDMA Map, 2016. However, there is no detailed information available regarding GIS data in Pakistan. For instance, the maps are not fully functional, and data sources are not available, who is the owner of the GIS data that was prepared for these projects? There are many reasons the GIS technology is not being appropriately used in Pakistan. First, the software is super expensive; second, the software does not provide national or local level general spatial data; and third, there is a substantial shortage of GIS professionals in Pakistan.

The policies of State Institutions

The current environmental policy of Pakistan called the NEP was approved in 2005. The policy aimed to manage natural resources and promote economic growth(The National Assembly of Pakistan, 1997). The NEP involves all the main sectors of the environment in its

strategy to progress the quality of life of the inhabitants. Some measures are taken to preserve the ozone layer by establishing the National Clean Development Mechanism (CDM) authority and promoting the use of environmentally friendly products. Many strategies are discussed in the NEP, but the financial means, financial provisions, and mechanisms of actions to implement these policies are not described in the policy. The policies that were prepared to protect Pakistan's human health, natural resources, and environment, are unrealistic and are not fully implemented so far at the local levels, because all the power remains under the control of the national government and they implement these policies without a financial mechanism at the top level.

According to the NEP, 2005, all the core environmental change policies will be executed at the national level by the MoE-Pakistan and the Pakistan Environmental Protection Agency (Pak-EPA). Although the Pakistan Environmental Protection Act 1997 gives all environment management authorities to provincial governments. Provincial governments must be in power in terms of implementing the policies. There has been very little coordination and association regarding the execution and progress of action plans between the central government agencies and the local government departments (International Monetary Fund, 2010). The policies need to be revised and included the proper role of the local governments with robust financial frameworks for the alleviation of the severe effects of climate degradation and for reducing the space between the federal administration and the local governments.

The Need for Local Government Involvement

It is necessary that climate change and climate-related disasters be dealt with at all levels but especially at the local levels. The NDMA was created in 2005 to help as a focal point for disaster management, emergency response, and response management in Pakistan. Then four PDMA was established in each of the four provinces of Pakistan to handle future disasters at the provincial levels. Unfortunately, in Pakistan, all the efforts of disaster management, environmental protection, and conservation of natural resources are being made at the top level so far. Since the NDMA and the PDMA's inception along with the national environmental protection institutions such as Pak-EPA and Ministry of Environment (MoE) have not been successful in implementing the NEP at the district, tehsil, union council, and community levels. As a whole, they have not strengthened their local institutions.

In Pakistan, local governments were created at three levels in 2001 through the announcement of the Local Government Ordinance 2001.

Fig. 6. Hierarchy of three levels of Local Governments.

Source: (Yazdani, 2003)

The district and city district levels are considered the highest level in the local governments in Pakistan, whereas Union, tehsil, and city town levels are considered the lowest level.

Climate change is likely to damage the environmental systems (watersheds, forests), social systems (health care and education centers), and infrastructure, including roads, power supplies, bridges, water, and sewage systems. Generally, these vulnerable and affected assets and infrastructures remain under the control of local governments. They are responsible for handling and delivering a range of quality services to their communities and have the experience to cope with the environmental variation at the local levels. Thus, local level administrations are the main element to achieve real results and solutions regarding environmental and natural resource protection because of three main reasons. First local authorities adopt their specific strategies, for instance, solid waste management rules and land administration regulations. Second, local government officials and employees can join neighborhood groups and organizations and can take affirmative actions to protect the environment and natural resources through lobbying practices. The ideas of other professional people and organizations can be added to the local decision-making processes through lobbying practices. The third reason is that cities have the depth of experience in the field of energy and waste management, public transport, and water supply and management(Linstroth & Bell, 2007).

As a whole, the disaster management and environmental protection institutions of the Pakistan government are using a top-down approach for disaster management and environmental conservation. That kind of approach does not work effectively. Due to the lack of connection and integrated and harmonious operation with the local governments, the NEP and NCCP police have not been successful in terms of reducing the impacts of climate change. The policies of the NEP, the NCCP, and the NDMA do not have accountability, local planning, or

financial frameworks. These kinds of aspects and angles in the policies where all the power remains under one agency cannot protect the economy, natural resources of the country, and the people from natural disasters.

IV. CONCLUSION

Pakistan has suffered most from the impacts of environmental change, which has posed threats to agriculture and food security, water security, and public health because of the weak institution system, unrealistic environmental policies, lack of environmental awareness among officers, and environmentally unsustainable practices. Effective, efficient, and realistic policies with financial accountability framework need to be prepared on an urgent basis for environmental protection and disaster management. The degradation of water resources, healthcare, and climate-related disasters have been happening every year due to climate change that is exacerbating the vulnerability of the country. There is a substantial nexus between air pollution and global warming and public health and disease mortality. Therefore, necessary preventive measures are required at a national level to reduced air pollution, which will bring public health benefits.

NCCP and NEP have a big gap between environmental change adaptation and actions between different levels of actions due to the lack of coordination among the local governments. It can be practical and realistic if the duties and tasks of local departments are defined clearly, and if the actions and financial, accountability, evaluation frameworks are constant. Additionally, climate change planning and judgments regarding environmental conservation should be realistic and take based on the facts through more muscular institutional coordination. Effective climate change adaptation measures require such leadership who supports decisions made at all local levels. The top-down approach of environmental change policy implementation does not work. The local government-based climate change programs' planning and implementation with effective financial management and accountability framework can better cope with the challenges of environmental degradation.

V. CONCLUSION AND RECOMMENDATIONS

Nowadays, environmental change has become an urgent issue for Pakistan because more severe climate-related disasters will damage its national resources, human health, food security, infrastructure, and human lives. In the light of uncertainty concerning future multispectral consequences of environmental change in Pakistan that have been discussed in the discussion section, multi adaptation policies and multi mitigation action plans should be taken to address environmental change.

The disaster management and environmental protection agencies of Pakistan will have to consider more to the climate change adaptation actions. First, the policy-makers will have to find the obstacles to adaptation and introduce reasonable and realistic environmental change policy measures that are real and applicable to Pakistan's environmental conditions.

The lack of awareness and closer collaboration has been a significant obstacle to environmental change adaptation. The environmental conservation awareness has now become essential for the citizens and the local officials of Pakistan to accomplish sustainable development and to protect the natural resources of Pakistan. The Pakistan government should establish a national adaptation council of action that would open for all the local governments, which would arrange a platform for closer collaboration, discussion, exchange of information and exchange of ideas on the environmental change adaptation policies and plans for the building environment. This platform would also provide a system for public involvement on the environmental change adaptation needs and plans.

The top-down approach of environmental change policy implementation does not work. The local government-based climate change program planning and implementation with effective financial management and accountability framework can cope better with the challenges of environmental degradation. The government should develop a tool for environmental assessment and awareness and capacity building of the officials and folks. Local governments and climate literate local officers can perform decisive front-line action to tackle environmental problems and natural resources management.

NCCP and NEP have a big gap between its environmental change adaptation and actions due to the lack of coordination among the local governments. The climate-related disasters and the government's climate change projects are managed and handled only at the national and rarely at the provincial levels. The top-down approaches are being used to control environmental change and natural disasters in Pakistan. This kind of approach cannot obtain the best results. The natural disaster management projects and climate change projects must be managed and handled at the local levels for obtaining the best results on time. The NDMA, the PDMA and the MoE in the NEP should clearly state the duties and

responsibilities of the local governments and enhance the participation of the local governments with more robust institutional coordination in management, and in the protection of the national resources and human health with realistic financial and accountability framework.

Many climate change adaptation policies of the national government have been unsuccessful in achieving the desired results because the policies do not comprise of negative and positive elements of environmental change for the vulnerable sectors that have affected and are affecting due to environmental degradation. For instance, the water sector and the agriculture sector in Pakistan are the most vulnerable sectors to environmental change that are stated in the NEP. Still, the NCCP does not define the harmful effects of environmental change on those sectors and does not state how environmental degradation will affect all aspects of water and agriculture. Such national environmental change policies to protect and manage the natural resources of Pakistan need to be rectified and rationalized.

The disaster management policies of the NDMA mostly focus on achieving short-term humanitarian relief goals and do not recognize climate change perspective. Particularly the disaster management policy of the NDMA is silent about the environmental change adaptation measures, although there is a substantial relation among disaster management and environmental adaptation. The NDMA and the MoE should include, recognize, and strengthen the coordinated method and incorporation of environmental change adaptation in its strategies and projects. They should establish a combined approach to combat environmental change and its risks by considering short to medium-term approach for disaster risk reduction, and short-term approach for disaster response and medium to long term approach for environmental change adaptation.

The central government should prepare practical polices to build household and commercial waste recycling centers for the local governments to collect and dispose of household and commercial waste. The local governments should be forced to turn to recycle waste properly as well as the local authorities need to take action for air pollution monitoring. The national government needs to set air quality and emissions standards to curb emissions on vehicles and power plants, on the use of biomass fuels and coal.

The bright and robust financial framework with the accountability model for environmental change adaptation projects should be established.

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The Combined Effect of Volume Water Supply and Varieties on Physiological Aspects, Growth, and Yield of Red Beetroot (*Beta vulgaris* L.) in Dryland Jatikerto, Indonesia

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***Abstract*—** *Increasing public awareness of living a healthy life, causing red beetroot demand in Indonesia has increased. However, with increasingly limited area of the plateau to the development of the plant, causing its development are directed to dry land are still many obstacles, such as limited availability of water for the plant level, and the high air temperature. Therefore, research that aims to obtain information about the right water needs and tolerant varieties on dry land needs to be done. A greenhouse experiment was conducted in UB's experimental station in Jatikertot village from June to September 2019. This study used a randomized complete block design (RCBD), consisting of 10 treatment combinations, namely the volume of water supply (350,550,750, 950, and 1150) mm water/season + varieties (ie Vikima and Ayumi), repeated 3 times. F test at 5% is used to determine the effect of treatments, while differences between treatments were referred to Honestly Significant Difference (HSD) value at 5%. The highest yield which includes chlorophyll a-b content, stomata density, root length, leaf surface area, total dry weight of plants and fresh weight of tubers/ plants was obtained in water supply volume of 1150 mm/season + Ayumi varieties. While the lowest was obtained in the volume of 350 - 550 mm water/season for both varieties for all variables observed.*

***Keywords*— Water requirements, Dry land, Red beet root varieties, Stomata density, Chlorophyll content.**

I. INTRODUCTION

Red beetroot (*Beta vulgaris* L.) is increasingly popular in Indonesia because its tubers can not only be used as raw materials for the cosmetics industry or natural dyes but more importantly, it can be used to maintain the health of the human body [18]. The extent of such utilization associated with full enough minerals such as potassium, magnesium, phosphorus, copper, and iron contained in the bulbs. Vitamins A, C, D, E, K, as well as protein, fat, carbohydrates, sugar, fiber, calories, and even betasianine and folic acid were also found in these tubers [25]. Due to the fairly complete content of nutrients and vitamins, red beetroot can be used to prevent cancer, reduce blood pressure and maintain heart health, help facilitate digestion, and weight loss [18]. Based on the importance of such utilization, the demand for red beetroot in Indonesia has increased by around 2 to 5%. However, the request can not be fulfilled because of the limited level of availability of these tubers.

Red beetroot are generally planted in the highlands which are marked by low temperatures, around 22.7 °C - 25.1 °C. On the other hand, the growth of horticultural commodities in Indonesia has increased significantly. As a result, competition in land use cannot be avoided. This is the reason for the limited circulation of bits in many traditional markets or supermarkets in Indonesia. Therefore, in an effort to increase the production of red beets, the development will be directed to dry land whose land is still quite large. However, with the many obstacles that must be faced in farming on dry land, causing this land cannot be fully utilized for agricultural activities [11]. One obstacle that must be faced in red beetroot cultivation in dry land is the high daily average air temperature which reaches 24ºC to 31ºC [21]. At high temperatures, as long as it is within the tolerance, they have a positive effect on plant growth and development. However, when the temperature has exceeded the tolerance limits, its impact on plant physiological disorders due to damage to the enzyme. High temperatures will also spur faster transpiration rates, and if not followed by adequate levels

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of water availability in the soil, plants will experience water stress. Moreover, one of the main constraints on dry land is the low level of availability of water for crops. In water stress conditions, the leaves of the plant appear to wither due to the inhibition of protein and chlorophyll biosynthesis. Water shortages also resulted in inhibition of initiation and differentiation on the apical meristem and cell enlargement, which leads to the inhibition of the development of roots, stem extension, and expansion of leaves [12]. The next impact is more rapid aging plant or senescense that causes the rate of photosynthesis decreases. Considering the important role of the water, research that aims to obtain precise information about the water requirements of red beetroot plants in dry land needs to be done.

Besides water, the selection of the right variety is also the key to the success of red beetroot cultivation in a dry land. This is because there is no precise information about the level of water requirements that are appropriate for red beetroot plants that are planted on dry land. Therefore, through this research, it is expected to be able to give the right answer about the level of water requirements that are appropriate for red beetroot varieties grown in a dry land. This information becomes important which is not only focused on the yield of tubers but also useful as a guideline in the management and maintenance of red beetroot plants in dry land. Basically every plant has a different character, and one of them can be expressed through the ability of varieties to adapt. For tolerant varieties, they will still be able to grow and develop normally even with new environmental changes. However, for sensitive varieties will show symptoms of withering as a result of the inhibition of protein and chlorophyll biosynthesis [19].

II. MATERIALS AND METHODS

2.1 Description of the study area

The greenhouse experiment has been carried out in the Universitas Brawiyaja Experimental Garden, located in Jatikerto Village, Malang Regency, Indonesia from June to September 2019. The location is located at an altitude of 330 m above sea level. Climatologically the average annual rainfall is around 1200 mm with an average daily temperature between 24ºC - 31ºC. The soil type is classified as Inceptisol with dusty loam texture with the proportion of sand (28%): dust (60%): clay (12%).

2.2 Research material

As planting material is Vikima and Ayumi's red beetroot seeds of varieties which has been aged 18 days after sowing. Polybags with a diameter of 20 cm as a place of planting, and dusty loam soil tekstur as planting medium. N fertilizer in the form of urea (46% N), phosphorus (SP_{36}: 36% P_2O_5), and potassium (KCl: 60% K_2O) respectively of 250 kg urea ha^{-1}, 50 kg SP_{36} ha^{-1}, and 50 kg KCl ha^{-1} were applied in this study.

2.3 Determination of field capacity

Measurement of field capacity in this experiment is necessary, due to the uniformity of the water content in each of the planting medium (polybags) on all treatments before giving water treatment applied. The method used is free drainage, which according to [10] the stages are as follows: First, the soil that will be used as a planting medium is crushed, then air-dried and then sieved with a sieve with a diameter of 3 mm. The sieved soil is then put into a polybag, as much as 8 kg/polybag, then filled with water until it drips. Soil that has been saturated is then stored for 2 x 24 hours until no longer dripping, and then weighed, expressed in weight of wet soil (WWS). The WWS is then oven at a temperature of 108ºC for 1 x 24 hours to get the soil dry weight (DWS). Field capacity (FC) is determined using the formula:

$$FC = \frac{WWS - DWS}{DWS} \times 100\%$$

2.4 Determination of water supply/day/polybag

Determination of water provision is based on (1) the value of crop coefficient (CC), (2) the value of HLO, and (3) the volume of water supplied/season (Table 1) [7]

Table 1. Water supply volume/day based on crop coefficient value, HLO value, and water supply volume/season

CC of red beetroot plant			**HLO value**	**Volume of water supplied (mm) / season**
Phase of plant growth	**Long phase of plant growth (days)**	**CC value**		
Initial growth	15	0.72	2.2 x 10^6 kg of soil / ha	350 mm/season
active vegetative	15	0.81	2.2 x 10^6 kg of soil / ha	550 mm/season
tuber formation	35	1.04	2.2 x 10^6 kg of soil / ha	750 mm/season
Tuber enlargement-harvest	15	0.70	2.2 x 10^6 kg of soil / ha	950 mm/season
Total		3.27	2.2 x 10^6 kg of soil / ha	1150 mm/season

Based on Table 1, it can be calculated the volume of water supplied /day/polybags with the following stages : (1) Convert the needs of water/season to hectares (eg 350 mm/season = 350 l/m^2 = 3.5 x 10^6 l/ha), (2) Determination of water/ polybag requirements is based on the calculation of water requirements/ha divided by the value of HLO, multiplied by the weight of the soil/polybag: [(3.5 x 10^6 l / ha) / (2.2 x 10^6 kg / ha)] x [8 kg] = 12.73 l / polybag. (3) Determination of water requirements/day/polybag for the initial growth phase is as follows: the results of point 2, multiplied by the result of dividing the value of CC (0.72) by the amount of CC

(3.27), then divided by the length of the growth phase (15 days): [(12.73 l / polybag) x [(0.72 / 3.27)] / 15 days = 0.18686 l /kg /polybag/day = 186.86 ml /polybag/day. Through the same calculation, then in Table 2 presented the volume of water given per plant/ day/ polybag which is based on the phase of plant growth, the value of CC and a long phase of growth.

Table 2. The volume of water supplied/day/polybags based on the volume of water supplied / season, plant growth stage, the value of CC and long phases of plant growth

Phase of plant growth	Long phase of plant growth (days)	The volume of water supplied (ml)/day/polybags based on the volume of water supplied / season (mm)				
		350 mm/season	550 mm/season	750 mm/season	950 mm/season	1150 mm/season
Initial growth	15	186.86 ml	293.33 ml	399.96 ml	506.73 ml	613.36 ml
Active vegetative	15	212.22 ml	333.33 ml	454.50 ml	575.83 ml	697.0 ml
Tuber formation	35	116.39 ml	182.86 ml	249.33 ml	315.89 ml	382.35 ml
Tuber enlargement-harvest	15	178.22 ml	280.0 ml	381.78 ml	483.70 ml	585.48 ml

2.5 Experimental design

Experiments using Randomized Complete Block Design (RCBD) and repeated 3 times. The combination of the volume of water supply and varieties as a treatment, consisting of 10 kinds, namely: (1) 350 mm water / season + varietas Vikima (P1), (2) 550 mm water/season + varietas Vikima (P2),(3) 750 mm water/season + varietas Vikima (P3), (4) 950 mm water/season + varietas Vikima (P4), (5) 1150 mm water/season + varietas Vikima (P5), (6) 350 mm water/season + varietas Ayumi (P6), (7) 550 mm water/season + varietas Ayumi (P7), (8) 750 mm water/season + varietas Ayumi (P8), (9) 950 950 mm water/season + varietas Ayumi (P9), (10) 1150 mm water/season + varietas Ayumi (P10). F test at 5% is used to determine the effect of treatments, while differences between treatments were referred to Honestly Significant

Difference (HSD) value at 5%. Regression analysis is used to explore relationships between two or more variables observed [9]

2.6 Research implementation

Transplanting is done when the seedlings have formed two leaves that have been fully open (around the age of 18 days after sowing) by placing one seed/polybag which has been filled with soil 8 kg and in conditions of field capacity. Phosphorus fertilizer was applied at the start of planting all doses, namely 0.23 g SP_{36}/polybag, while nitrogen and potash fertilizers respectively of 1.14 g urea/polybag and 0.23 g KCl/polybag be granted gradually. Phase I is applied when the plant was 7 days after planting (dap) 1/3 of the dose, and the remaining 2/3 was applied at 14 daps. Fertilizer is applied to each hole of fertilizer with a depth of 7 cm and a distance of 5 cm from the plant and then covered with compost. The application of the water supply is done after the plant was 14 days after planting with the assumption that plants has been able to adapt to their environment.

2.7 Data collection

Observation's physiological aspects that include the measurement of chlorophyll-a ,b and stomata density is done at the maximum vegetative phase (40 daps). As for agronomic observations which include measurements of root length, leaf surface area, total dry weight of plants, and fresh weight of tubers/plants carried out destructively at harvest (60 daps) by taking four samples per treatment.

2.7.1 Chlorophyll content

Measurement of chlorophyll content refers to methods spectrophotometer, which according [3] are as follows:

Chlorophyll -a: (12.21 x λ 663) – (2.81 x λ 646),

Chlorophyll –b: (20.13 x λ 646) - (5.03 x λ 663).

2.7.2 Stomata density

Measurement of stomata density using a microscope with magnification 40 times to obtain a clear visualization of stomata images. The stages of implementation according [6] to are as follows:

1. Printing stomata at 11:00 AM with transparent nail polish on the leaf surface, and slowly lift it when it is dry
2. Stomata mold that has been lifted from the surface of the leaf, and then put in a plastic bag that had been labeled treatment
3. Stomata observed with a microscope with a magnification of 40 times, until a clear visualization of stomata
4. Counting the total number of stomata observed in a wide field of view (ie: 257 µm x 345 µm), or equal to 0.088 mm^2
5. Determine the density of stomata using the formula [14] :

$$\text{Stomata density} = \frac{\text{Number of stomata}}{\text{Unit area of field view}}$$

2.7.3 Root length

Root length is measured from the root tip to the base of the stem, using a ruler

2.7.4 Leaf surface area

Leaf surface area was measured using a leaf area meter type LI-3100 C for leaves that had been fully opened, excluding young or senescence leaves. Leaf samples are placed above the glass lens in a non-folded or non-overlapping position. Records were taken for all sample leaves from four sample plants per treatment, then averaged. The leaf surface area value is determined by multiplying the average value of the recording by the correction factor. Correction factors can be sought by dividing the measurement value of the actual paper area (for example 100 cm^2) with the value of the paper area that has been measured by leaf area meters, for example 99 cm^2. So the value of the correction factor is 99 cm^2/100 cm^2 = 0.99 [24]

2.7.5 Total dry weight of plants

Measurement of the total dry weight of plant by using oven-type OVL 12 with a temperature of 81ºC. Before drying, roots, stems, leaves, and tubers should be separated, because every part of the plant requires different drying time to achieve a constant dry weight. The plant parts that have been separated then put into a cement bag, and then put into the oven. Weighing is done after a constant dry weight is achieved, then added up [24].

2.7.6 Fresh weight of tubers/plant

Fresh weight of tubers/plant obtained by weighing the tubers after being separated from the roots and leaves by using an analytical balance Scout- pro type.

III. RESULT AND DISCUSSION

3.1 Result

Measurement of physiological aspects is carried out when the plant is 40 days after planting with the consideration that the plant has entered the maximum

vegetative phase. While the agronomic observations were made at harvest time (60 daps)

3.1.1 Physiological aspects

3.1.1.1 Chlorophyll content

There was a significant effect at p = 0.05 of the combination treatment of water supply and varieties on the measurement of Chlorophyll a, b content at 40 dap (Table 3).

Table 3. The average chlorophyll a and b content in various treatment combinations of water supply and varieties at the age of 40 dap

Treatment	Code	Average chlorophyll content (µg/2g fw)	
		Chlorophyll-a	Chlorophyll
350 mm of water/ season + Vikima varieties	P1	11.63 a	0.84 a
550 mm of water/ season + Vikima varieties	P2	16.18 abc	2.47 b
750 mm of water/ season + Vikima varieties	P3	16.68 c	2.55 b
950 mm of water/ season + Vikima varieties	P4	17.07 cd	4.06 cd
1150 mm of water/ season + Vikima varieties	P5	30.09 e	4.30 d
350 mm of water/ season + Ayumi varieties	P6	11.96 a	1.14 a
550 mm of water/ season + Ayumi varieties	P7	15.68 abc	1.45 a
750 mm of water/ season + Ayumi varieties	P8	16.67 bc	2.45 b
950 mm of water/ season + Ayumi varieties	P9	21.50 d	3.44 c
1150 mm of water/ season + Ayumi varieties	P10	31.44 e	4.50 d
BNJ 5%		4.68	0.80

Note: Numbers are accompanied by the same letters in the same column are not significantly different by HSD 5%. dap: days after planting, fw : fresh weight

Table 3 shows that the higher chlorophyll-a content was obtained in treatments P5 and P10 when compared to other treatments, respectively 30.09 and 31.44 µg / 2g fw, and both showed no significant differences at p = 0.05. While the lower one was obtained in the treatments P1, P2, P6 and P7, respectively 11.63, 16.18, 11.96, and 15.68 µg/2g fw, and the four treatments showed no significant difference at p = 0.05. However, for the P2 and P7 treatments, chlorophyll-a is generated also not significantly different with treatment P3, P4 and P8, respectively 16.68, 17:07, and 16.67 µg /2g fw. Although the three treatments are still able to produce chlorophyll-a higher compared to the treatment P1 and P6, but lower when compared to the treatment of P5,P9 and P10. The chlorophyll-a content also showed no significant difference at p = 0.05 in P4 and P9 treatments, respectively 17.07 and 21.50 µg/2g fw. As for **chlorophyll-b**, the lower results obtained in the treatment of P1, P6, and P7, respectively 0.84, 1:14, dan1.45, and the three treatments showed no significantly different at p = 0.05. In treatments P2 (2.47 µg/2g fw), P3 (2.55 µg/2g fw), and P8 (2.45 µg/2g fw), the chlorophyll-b content produced was also not significantly different at p = 0.05. However, these results are still higher than treatment P1, P6, and P7, but lower compared to the treatment P4, P5, P9 and P10 that has reached 4.06, 4.30, 3.44, and 4.50 µg/2g fw. Higher chlorophyll-b content was obtained in treatments P5 and P10, although not significantly different from treatment P4.

3.1.1.2 Stomata density

There was a significant effect at p = 0.05 of the combination treatment of water supply and varieties on the measurement of stomata density at the age of 40 days after planting (Table 4).

Table 4. Average stomata density in various treatment combinations of water supply and varieties at the age of 40 dap

Treatment	Code	Stomata density (field of view (257 x 345 / cm^3) / mm^2	Category
350 mm of water/ season + Vikima varieties	P1	111.74 a	Low
550 mm of water/ season + Vikima varieties	P2	123.11 a	Low
750 mm of water/ season + Vikima varieties	P3	172.35 bc	Low
950 mm of water/ season + Vikima varieties	P4	192.23 c	Low
1150 mm of water/ season + Vikima varieties	P5	264.20 d	Medium
350 mm of water/ season + Ayumi varieties	P6	117.42 a	Low
550 mm of water/ season + Ayumi varieties	P7	135.42 abc	Low
750 mm of water/ season + Ayumi varieties	P8	176.14 c	Low
950 mm of water/ season + Ayumi varieties	P9	257.58 d	Medium
1150 mm of water/ season + Ayumi varieties	P10	284.09 d	Medium
BNJ 5%		48.08	

Note: Numbers are accompanied by the same letters in the same column are not significantly different by HSD 5%. dap: days after planting.

Table 4 shows that the lower stomatal density generated by the treatment of P1, P2, P6, and P7, respectively amounted to 111.74, 123.11,117.42, and 135.42/mm^2, and the fourth treatment showed no significantly different at p = 0.05. However, for the treatment of P7, the resulting stomatal density is not significantly different from the treatment of P3, P4, and P8, respectively 172.35,192.23,176.14/mm2, but lower when compared to the treatment of P5, P9 and P10. In treatments P5, P9, and P10 the highest stomata density was obtained compared to the other treatments, each of 264.20, 257.58, and 284.09 / mm^2, and all showed no significant difference at p = 0.05. Fig. 1 to 10 present the levels of stomata density from various combinations of volume water supply + varieties.

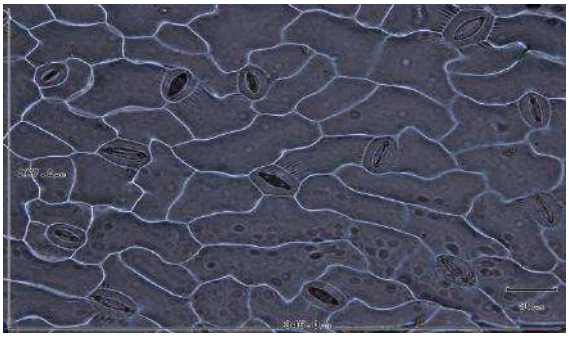

Fig.1: 350 mm water supply / season + Vikima varieties (P1)

Fig.2: 550 mm water supply / season + Vikima varieties (P2)

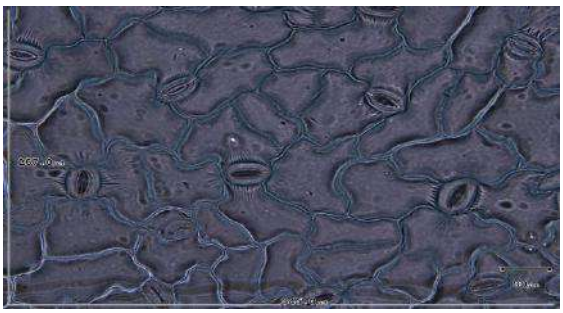

Fig.3 : 750 mm water supply / season + Vikima varieties (P3)

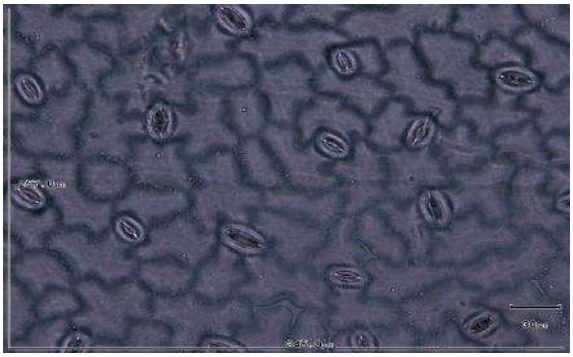

Fig.4 : 950 mm water supply / season + Vikima varieties (P4)

Fig.5 : 1150 mm water supply /season + Vikima varieties (P5)

Fig.6 : 350 mm water supply / season + Ayumi varieties (P6)

Fig.7 : 550 mm water supply / season + Ayumi varieties (P7)

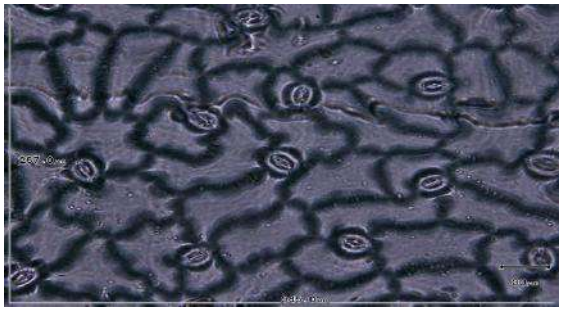

Fig.8 : 750 mm water supply / season + Ayumi varieties (P8)

Fig.9 : 950 mm water supply / season + Ayumi varieties (P9)

Fig.10 : 1150 mm water supply /season + Ayumi varieties (P10)

3.1.2 Agronomic observations

3.1.2.1 Root length, Leaf surface area, Total dry weight of plants

There was a significant effect at p = 0.05 of the combination treatment of water supply and varieties on the measurement of root length, leaf surface area, total dry weight of plants at harvest as presented in Table 5.

Table 5. The average root length, leaf surface area, total dry weight of plants in various combination treatment of water supply and varieties at harvest (60 daps)

Treatment	Code	The average root length, leaf surface area, total dry weight of plants at harvest		
		Root length (cm)	Leaf surface area(cm^2)	Total dry weight (g)
350 mm of water/ season + Vikima varieties	P1	5.33 a	452.82 a	9.36 a
550 mm of water/ season + Vikima varieties	P2	7.17 ab	564.83 ab	12.03 a
750 mm of water/ season + Vikima varieties	P3	9.00 bcd	795.85 bcd	18.08 b
950 mm of water/ season + Vikima varieties	P4	9.67 cd	858.80 d	20.89 b
1150 mm of water/ season + Vikima varieties	P5	10.17 d	1357.86 e	26.78 c
350 mm of water/ season + Ayumi varieties	P6	6.67 a	520.24 a	10.89 a
550 mm of water/ season + Ayumi varieties	P7	7.33 ab	559.43 ab	11.22 a
750 mm of water/ season + Ayumi varieties	P8	9.00 bcd	843.43 cd	19.60 b
950 mm of water/ season + Ayumi varieties	P9	10.0 d	1262.35 e	21.32 b
1150 mm of water/ season + Ayumi varieties	P10	12.5 e	1797.05f	35.54 d
HSD 5%		2.28	254.27	5.18

Note: Numbers are accompanied by the same letters in the same column are not significantly different by HSD 5%. dap: days after planting.

Table 5 shows that shorter roots were obtained at treatments P1, P2, P6, and P7, respectively 5.33, 7.17, 6.67, and 7.33 cm. Although for the treatment P2 and P7, root length produced is not significantly different at p = 0.05 with treatment P3 and P8, respectively along 9 cm. While the root length produced by the treatment is also not significantly different from the treatment P4, P5, and P9, respectively along 9.67, 10:17, and 10.0 cm. Although the root length generated by the third treatment is still longer when compared with the treatment P1, P2, P6, and P7.The longest root was obtained at treatment P10, which is 12.5 cm.

Table 5 also shows that a narrower leaf surface area was obtained in treatments P1, P2, P6, and P7, and the four treatments showed no significant difference at p = 0.05. The leaf surface area was 452.82, 564.83, 520.24 and 559.43 cm^2, respectively. However, for P2 and P7 treatments, the leaf surface area produced was not significantly different from P3 treatment, which was 795.85 cm^2. While for the P3 treatment, the leaf surface area produced was also not significantly different from the P4 and P8 treatments, respectively 858.80 cm^2 and 843.43 cm^2. While for the P3 treatment, the leaf surface area produced was also not significantly different from the P4 and P8 treatments, respectively 858.80 cm^2 and 843.43 cm^2, although these results were still higher than the treatments P1, P2, P6, and P7. Treatment of P5 and P9, leaf surface area produced was not significantly different at p = 0.05, but it was wider when compared to other treatments, except for treatment P10. The most extensive leaf surface was obtained in treatment P10, which is an area of 1797.05 cm^2. In the measurement of the total dry weight of plants, the highest yield was obtained in treatment P10, which amounted to 35.54 g / plant, then followed by treatment P5, which amounted to 26.78 g / plant. While the lower one was obtained at treatments P1, P2, P6, and P7, respectively 9.36, 12.03, 10.89, and 11.22 g / plant. The same thing was also found in the treatments of P3, P4, P8, and P9, respectively 18.08, 20.89, 19.60, and amounting to 21.32 g/plant. Table 5 can also be explained that the volume of water supplied various varieties Ayumi able to provide better results than Vikima varieties. Although statistically, significant differences do not occur in all treatments for water treatment, except for treatments P5 and P10. However, there is a tendency that the value of Ayumi generally higher than Vikima varieties at various observation variables such as root length, leaf surface area, as well as the total dry weight of the plant. Differences in response to both varieties as shown in Fig. 11 to 13.

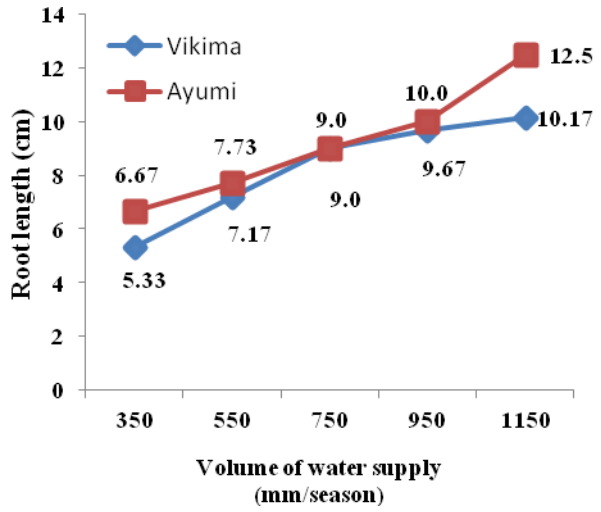

Fig.11: The response of the two red beetroot varieties to the root length at various volumes of water supply

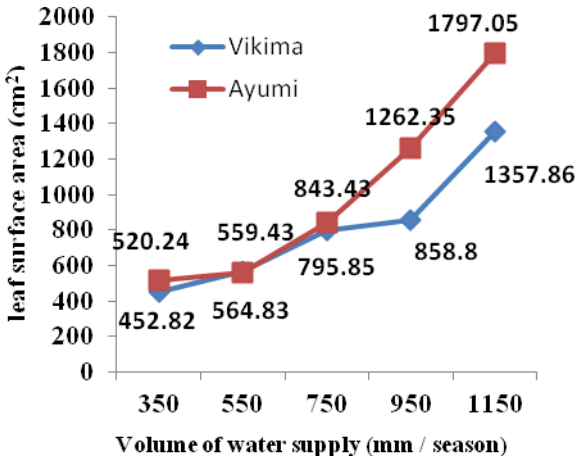

Fig.12: The response of the two red beetroot varieties to the leaf surface area at various volumes of water supply

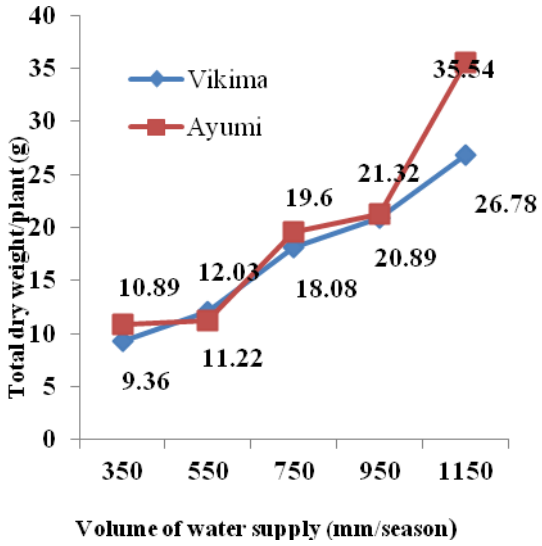

Fig.13: The response of the two red beetroot varieties to the total dry weight of plant at various volumes of water supply

3.1.2.2 Tuber fresh weight/plant

Fresh weight of tubers / plant affected by the treatment of the water supply volume and varieties. Average fresh weight of tuber / plant at different volume of water supply and varieties are presented in Table 6.

Table 6. Average of tuber fresh weight/ plant in various combination treatment of water supply and varieties at harvest (60 daps)

Treatment	Code	Tuber fresh weight/plant (g)	
350 mm of water/ season + Vikima varieties	P1	29.70	a
550 mm of water/ season + Vikima varieties	P2	61.10	c
750 mm of water/ season + Vikima varieties	P3	87.67	d
950 mm of water/ season + Vikima varieties	P4	104.09	d
1150 mm of water/ season + Vikima varieties	P5	144.76	e
350 mm of water/ season + Ayumi varieties	P6	34.31	ab
550 mm of water/ season + Ayumi varieties	P7	57.46	bc
750 mm of water/ season + Ayumi varieties	P8	98.31	d
950 mm of water/ season + Ayumi varieties	P9	111.47	d
1150 mm of water/ season + Ayumi varieties	P10	193.78	f
HSD 5%		25.20	

Note: Numbers are accompanied by the same letters in the same column are not significantly different by HSD 5%. dap: days after planting.

Table 6 shows that the highest fresh weight of tubers / plants was obtained at treatment P10, which was 193.78 g / plant, compared to other treatments. While the lower one was obtained at treatments P1 and P6, respectively 29.70 and 34.31 g / plant. Although for the P6 treatment, the fresh weight of the tubers produced did not differ significantly at p = 0.05 with the P7 treatment (ie 57.46 g / plant), and the treatment was also not significantly different from the P2 treatment, which was 61.10 g /plant. fresh weight of tuber/plant showed no significantly different at p = 0.05 for the treatment P3, P4, P8, and P9, respectively by 87.67, 104.09, 98.31 and 111.47 g / plant. However, the four results are still higher than the treatments P1, P6, P2, and P7, although lower than the P5 and P10 treatments which have reached values of 144.76 and 193.78 g / plant. On the other hand to evaluate the response of each variety to the fresh weight of tubers/plants in various water distributions is presented in Fig. 14.

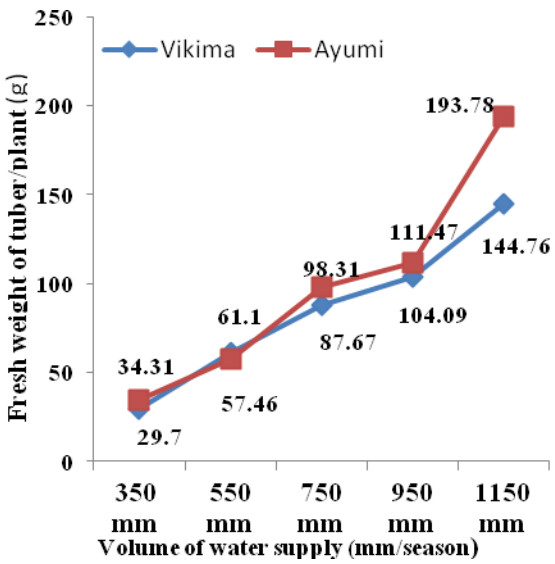

Fig 14: The response of the two red beetroot varieties to the fresh weight of the tuber/plant at various volumes of water supply

3.2 DISCUSSION

3.2.1 Chlorophyll content

Measurement of chlorophyll content can be used as an indicator to determine crop water shortages. The results showed that the higher content of chlorophyll-a ($C_{55}H_{72}O5N_4Mg$) and chlorophyll-b ($C_{55}H_{70}O_6N_4Mg$) was

obtained in treatments P5 and P10. While the lower one was obtained in treatments P1 and P6 (Table 3). The lower chlorophyll content produced by treatments P1 and P6 as a result of the low level of water availability for plants due to the low volume of water supplied. In limited water conditions, there is an inhibition in the process of nitrification. As a result, the process of conversion of nitrite (NO_2^-) to nitrate (NO_3^-) is disturbed that causes N to be less available for plants. On the other hand with low levels of water availability also causes low levels of nutrient solubility (especially elements N, and Mg). This has led to low uptake of these elements when the two elements are very important in relation to the preparation of chlorophyll [1]. Thus it can be said that for plants that are in limited water conditions, chlorophyll biosynthesis is inhibited. Chlorophyll biosynthesis inhibition was also fueled by the low availability of carbohydrates, conditions of temperature and light absorption [8]. At high-temperature conditions such as in the study site, it will spur an increase in the rate of evapotranspiration which causes plants to experience a water deficit, especially for plants that are only watered as much as 350-550 mm/season. The incidence of wilting is unavoidable, and this is one of the physiological responses of plants that lack water. As a result, the rate of photosynthesis decreases, and this decrease has an impact on the low assimilate produced. Though this assimilate is needed in chlorophyll biosynthesis. Hence, when crops suffer from lack of water, chlorophyll biosynthesis and carbohydrate disturbed. As a result, the amount of chlorophyll formed is also low, as presented in Table 3. These results are in line with [13] who found that the ratio of chlorophyll a, b in plants that lack water is lower (ie, 2.16), compared to the control treatment, which reached 3.29 These results are also supported by the high value of R^2 generated from both the regression equation (Fig 15). The resulting linear form of the equations illustrates that the level of chlorophyll content that forms is determined by the extent of the water available for plants.

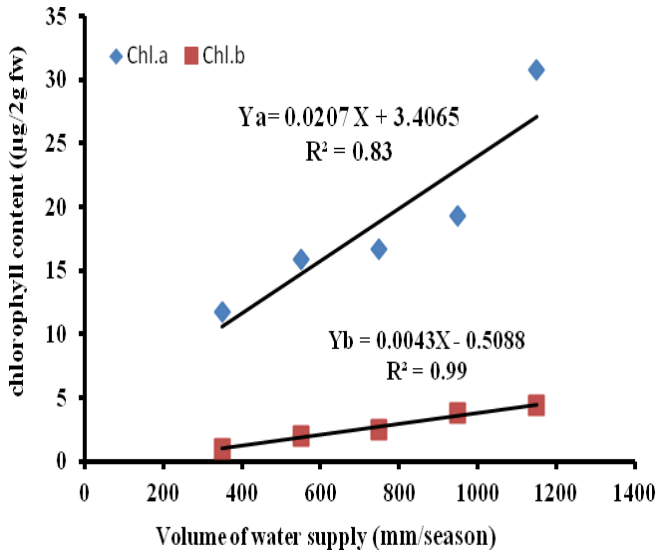

Fig. 15. The relationship between the volume of water supply and the chlorophyll content

3.2.2 Stomata density

Table 4 shows that a higher density of stomata were obtained in the treatment of P5, P9 and P10. While the lower was obtained in treatment P1, P2, P6, and P7 (Table 4). The lower density of the stomata is closely related to the efforts of plants to reduce more water loss, especially through the process of transpiration when water is limited. [14] stated that some important strategies developed by plants when subjected to drought are to reduce the number and density of stomata, and stomatal closing quickly. This aims to reduce the amount of water loss from the body of the plant through the process of transpiration. This is in line with the results of research by [4] who informed that the transpiration rate in maize decreased by around 54.21% when it was only watered by 30-40% of field capacity. However, it then showed an increase of 30.24% (from 30-40% of field capacity) when it was irrigated as much as 70-80% of field capacity. This means that the decline in the rate of transpiration is closely linked to the low density of stomata that are generated, and the high number of stomata that experience closure. On the other hand, the closing of stomata is also caused by increased synthesis and release of abscisic acid (ABA). The presence of ABA in the root is caused by an increase in pH in the root xylem vessels. As a result, there is an ABA flow from the roots to the shoots (leaves), and ABA accumulation in the leaves causes stomata to close [26]. This statement is reinforced by the results of stomata density measurements as presented in Figures 1 to 10. The figure shows the difference in stomata density between treatments. In the provision of high water, ranging from 750 to 1150 mm/season, both in the Vikima and Ayumi varieties (P3-P5 and P8-P10), the level of

stomata density produced is higher than P1, P2, P5, and P6. However, there is a tendency that the level of stomata density in the Ayumi variety is higher than that of the Vikima variety in various volumes of water supply. This indicates that the Ayumi variety is more adapted than the Vikima variety.

3.2.3 Root length

Drought stress is a term used to state that plants suffer from lack of water due to the limited water from their environment, especially in their growing media. The results showed that the longest root was obtained in the P10 treatment. Whereas the shorter one was found in the treatments P1, P2, P6, and P7 (Table 5). The shorter roots produced by the four treatments are as a result of (1) the low content of chlorophyll a and chlorophyll b, (2) the low level of stomata density, all of which are caused by low levels of water availability for plants. Water for plants not only serve as a solvent, but also plays a role in regulating the opening and closing of stomata. Stomata will open when turgor pressure from both guard cells increases. While increasing the turgor pressure of the guard cells is determined by the entry of water into the guard cells. When guard cells take water through osmosis, guard cells will swell causing stomata to open. The stomatal opening will soon spur the photosynthetic activity when other factors are also met. Therefore, plants that suffer a lack of water will have an impact on the low assimilate due to closed stomata. As a result, the rate of plant growth decreases, including the process of root development [26]. The shorter roots are also caused by the high obstacles that must be passed by the roots due to quite dense and hard growing media used, so it takes a lot of energy. Meanwhile, assimilate available low. As a result, the roots are not able to develop normally. [5] also found that black potato plants were irrigated as much as 300 mm/season, root length produced shorter, that is 42.40 cm, while for crops to be irrigated as much as 1200 mm/season, root length output reached 52.54 cm. Fig. 11 shows that the Ayumi variety has a better level of adaptability than the Vikima variety at various volumes of water supply. Thus, the length of the root produced is generally longer than the Vikima variety

3.2.4 Leaf surface area

Leaves are photosynthetic organs for plants. Therefore, leaf surface area needs to be observed in this study. The results showed that the widest leaf surface was found in P10 treatment, which was 1,797.05 cm^2 (Table 5). This is due to the higher chlorophyll content and the longer root formed (Table 3.5). The high chlorophyll content will spur the rate of photosynthesis due to more light that can be captured by the leaf chlorophyll. In addition, the absorption of nutrients by the roots runs normally because the roots are able to develop normally. Considering the growth and development of plants requires a certain amount of energy, the energy that formed in the form of assimilates will be used for this process, including the expansion of leaves. However, the increase in the leaf area is not always followed by the increase in the total dry weight of the plant. The regression analysis proved the quadratic relationship between leaf area (X) with a total dry weight of plants (Y) given by an equation: $Y = - 0015\ X^2 + 47.38\ X - 9347.9$; $R^2 = 0.99$. This equation shows that with increasing leaf area to its optimum limit, it is still followed by an increase in the total dry weight of plants. However, an increase in leaf area above the optimum causes a reduction in the total dry weight of the plants produced. Based on these equations, it can be determined the optimum leaf area for beet plants planted in dryland is 1,579.33 cm^2 with a maximum total dry weight of 28.07 g / plant. Conversely, if crops suffer from lack of water, whether caused by the low volume of water provided and because of the dry conditions of the atmosphere, causing a lack of imbalance between the rate of evapotranspiration and water availability levels. As a result, crops suffer from water stress and result in physiological and morphological changes that may occur at the molecular and cellular levels [16]. Physiological and morphological changes are shown as a result of water stress is (1) a decrease in the rate of growth of plants due to the low assimilate produced, (2) impaired and chlorophyll biosynthesis inhibition due to lower N and Mg uptake by plants is a constituent element klorofil [1]. (3) The reduction of leaf surface area serves to keep the cell potential remains high. In this condition, cell turgidity remains high, so the rate of water loss can be minimized. This was also proven in this study which found that the narrowest leaf surface area was produced by treatments P1 and P6, where the water provided was the lowest, ie 350 mm/season (Table 5). Reduction in leaf surface size is actually a response of plants when experiencing water shortages, namely by changing the new assimilate distribution, from leaves to roots. Besides aiming to control the high rate of transpiration. The purpose of the change is to support root growth in an effort to increase the capacity of water absorption by plant roots [22]. Fig. 12 shows that the Ayumi variety showed better growth than the Vikima variety in various water supplies. This indicates that the Ayumi variety is more tolerant than the Vikima variety.

3.2.5Total dry weight of plants

In this study, the highest total dry weight of plants was obtained in the P10 treatment, amounting to 35.54 g / plant. While the lower is obtained in the treatment of P1, P2, P6,

and P7 (Table 5). The lower total dry weight of the plants produced is related to (1) lower chlorophyll a-b formation (Table 3), (2) narrower surface area of the leaves produced (Table 5), (3) shorter roots (Table 5), and (4) lower levels of stomata density produced (Table 4). When the four factors necessary to the plant is limited, then the limitations that will restrict the growth and development of plants [15, 1]. While the total dry weight of plants is a function of growth, and when the plant growth process is disrupted, the total dry weight of the plants produced is also low due to the non-maximum function of each plant organ [23]. These results concur with those of [20] were informed that the taro plants watered as much as 500 mm of water/season, total dry weight of plants produced amounted to 9.53 g / plant, and this value is 73.95% lower than the with irrigated as much as 1000 mm/season reached 36,59g/ plant. Regression analysis also proved the existence of a linear relationship between water requirements (X) and total plant dry weight (Y) through the following equation: $Y = 0.014 X + 0.08$, $R^2 = 0.94$. The high coefficient of determination which reached 94 indicates that the total dry weight of plants, 94% is determined by wate Fig. 13 shows that in general, the total dry weight of plants produced by the Ayumi variety is higher at various volumes of water supply than the Vikima variety. This indicates that the Ayumi variety is more adaptive than the Vikima variety.

3.2.6 Tuber fresh weight / plant

The economic yield of red beet plants in the form of tubers, and based on this study it was found that the highest fresh weight of tubers/plants was obtained at P10 treatment, which was 193.78 g / plant. While the lower was found in treatment P1 and P6 (Table 6). The lower tuber weight is due to the lower content of chlorophyll a-b produced (Table 3), the lower density of stomata formed (Table 4), the shorter root produced (Table 5), the narrower surface area of leaves (Table 5)), all of which are important factors and determine the development of a plant. As a result, the physiological processes of plants are disturbed, especially photosynthesis. Obstructed and disruption of this process will result in a low assimilate formed. Whereas assimilate is the main energy source for the plant. [14] states that the energy that is formed will be used for energy growth, as energy reserves, and partly to be stored in the sink which is an economic yield of plants (tubers). Given the importance of the assimilate function, it causes the plants to have limitations as mentioned above, resulting in disruption of plant physiological processes, especially photosynthesis which finally has disrupted the process of forming and filling the bulbs [2]. It becomes reasonable when treatment P1 and P6, the fresh weight of tuber/plant produced low. In addition, the tuber weight is also determined by the level distribution of assimilates from the source to the sink (bulb) can be measured through a formula EY/TDW [21]. EY is tuber dry weight, while TDW is total plant dry weight, and based on this study it was found that higher EY / TDW was obtained in treatments P5 and P10, respectively 64.04% and 65.31%. This indicates that approximately 64-65% of the total assimilated generated has been allocated to the tuber. As for the P1 treatment only reaches 43.06% and amounted to 50.81% for the treatment of P6. This has led to a lower fresh weight of tubers/plant on care P1 and P6 respectively reaching only 29.70 and 34.31 g / plant. These results concur with those of [20] on taro plants which showed that the irrigation of 500 mm/season, the EY/TDW only reach of 0.12%, while the irrigation of 1,500 mm/season, the EY/TDW irrigation reaches approximately 0.19%. Fig.14 shows that the Ayumi variety has a better response to various water supply volumes to the fresh weight of tubers/plants than the Vikima variety. Although statistically, not all treatments are able to produce significant differences. However, the fresh weight of tuber/plant produced by Ayumi varieties has a higher tendency than Vikima varieties.

IV. CONCLUSION

Based on these results, it can be concluded that in order to get the best plant growth, the highest yield, as well as the best physiological aspects of red beet plants planted in the dry land, it needs irrigation as much as 1150 mm of water/season by using the Ayumi variety (P10).

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AUTHORS CONTRIBUTION

Nur Edy Suminarti as the main researcher, contributed to the design of this study, starting from the determination of

treatment, experimental design, stages of observation, and writing of this manuscript.

Tika Noviana Dewi as a research assistant, contribute in determining the location of research, implementing the research in the field and help the observations in the field

Aninda Nur Fajrin as a research assistant, contributed to data analysis and data collection on observations

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Gender Inequality in Nepalese Agriculture: Issues Concerning Sustainability and Food Security

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Abstract*— *Despite of the advancement in technology and excessive use of chemical fertilizers for agricultural production, about 870 million people around the globe are hunger stricken due to various resource scarcities. There is a need to boost the agricultural productivity in order to feed the population which is growing in sky rocketing way. Food insecurity is a burning problem affecting nutrition, health and the betterment of population across the globe. Basically, food security depends on four pillars: availability, access, utilization and stability. Sustainable approaches in agricultural sector are of great importance to improve the food productivity and security along with mitigating nutrition problem around the world. In industrialized countries, less developed and developing countries, gaps in earnings by gender have long been evident. For the equivalent kind of work, women wages are found to be 60-75% of men wages. The agricultural sector of Nepal contributes 28 percent to national GDP and this share tends to rise in the future years. At present the productivity of Nepal is sufficient to feed its population but there are a lot of problems related to gender inequality which eventually affects food and nutritional security. Several such aspects are discussed in this paper.

Keywords*— *Food Security, Gender inequality, Productivity, Sustainability.

I. INTRODUCTION

The mankind's dominion of planet earth came into existence by the revolutionary shift in the provision of food from hunting and gathering to agriculture (Helms, 2015). A frequent doubt voiced to the carrying capacity of the earth because of the rapidly growing population and limited resources has been a major point of concern of today's world (Smil, 1994).The livelihood of the majority of Nepalese population is dependent upon agriculture and livestock. As a major fraction of the country's population resides in the rural villages, agriculture and live stock has been established as a major source of income to the people. The agriculture sector of Nepal is found to mostly rely on the traditional farming and cultivation system. As per Central Bureau of Statistics (CBS, 2011), 83 percent of the population depends on agriculture and the same population resides in a rural area. The agricultural sector contributes 28 percent to national GDP (NRB, 2018); Chaudhary, 2018).

According to the International food policy research institute (IFPRI, 2011) the agriculture system must improve their sustainability and maintain its own viability by using techniques that allow for the continual reuse so as to ensure the food security. The concept of suitability is related to the potential for long term maintenance of wellbeing, which has ecological, economic, political and cultural dimensions (Smith and McDonald, 1998). The agriculture sector in Nepal is facing increasing obstacles from different sectors in order to meet the regular needs (CBS, 2011). The unsustainable environment in agriculture and food systems is the impact of gender inequality and lack of long-term maintenance and utilization of the resources. The word sustainable means pertaining to a system that maintain its own viability. Moreover, sustainability means keeping an effort going continuously or we can say the ability to last out and keep from falling.

For growth and maintenance activities, an individual requires energy and nutrients which is supplied by food. As per the United Nations committee on world food security, food security means that all people, at all-time have physical, social and economic access to sufficient, safe and nutritious food that meets food preferences and dietary needs for an

active and healthy life.An average food energy availability of 2,900 kcal is considered satisfactory (Gilland, 2002). The current challenge to feed 7.6 billion people globally with limited and gradually decreasing cultivable land and food produce has ultimately posed the risk to food security. The global population is to be expected to rise to 9 billion by 2040 and if the growth rate remains the same, it will rise to 11 billion by 2100 (World Population Clock, 2015). So, the problem of food security is a burning issue for the developing countries like Nepal.

By now, the total crops produced in Nepal is enough to feed everyone residing in the nation. With the growing population and static productivity, the food security issue has evolved to a major concern. The cause of the lower food productivity and increasing issues of food security could be gender inequality and imbalance in the agriculture sector.

II. AGRICULTURE SUSTAINABILITY AND FOOD SECURITY

Agriculture is one of the most important practices developed by human race for their survival and proliferation on this planet. Because of agriculture, humans were able to shift their life style from Stone age and settled in colonies now we know as cities and villages. However, the growing population and limited resources lead to the invention and application of chemical fertilizers and pesticides which boosted the agricultural productivity initially (Arora, 2018). Due to the over use of chemical fertilizers and pesticides, the natural productivity of soil in the several regions of Nepal has decreased drastically which is posing a threat to food productivity, sustainability and eventually to the food security.Despite of the advancement in technology and excessive use of chemical fertilizers and pesticides for agricultural production, around 870 million people are hunger stricken due to various resource scarcities. There is a need to boost the agricultural productivity by 60% if we want to fulfill the need of 9 billion population in 2050(FAO,2012).Over use of chemical substances in agriculture sector has also led to severe environmental problems like pollution of soil, water and air, development of pesticide resistant organisms, loss of productivity of soil, and now is leading to serious threats to food security, biodiversity and human health (Aktar, 2009).

Sustainable approaches in agricultural sector are of great importance to improve the food productivity and security along with mitigating nutrition problem around the world.Basically,food security depends on four pillars: availability, access, utilization and stability. Food and agricultural systems overlap not only in the area of food production, but also in the diverse areas like processing, transportation, marketing and many more (Capone et. al ,2014). Sustainability in Agriculture can be achieved by planning, utilizing and implementing the techniques of farming which would increase crop productivity so as to meet demands of growing population along with conserving and protecting environment and its natural sources (Arora, 2018). In order to achieve sustainability, upliftment of economic growth is necessary which has a direct link in achieving food security, improving nutrition and sustainable agriculture (SDGUSA, 2018).According to Chauhan et.al (2017),for ensuring food security and sustainability in agriculture, the traditional biological methods should be used by amalgamating it with recent innovations in biotechnology and bio-engineering, so as to utilize the genes of useful microbes and their metabolites as well as improving the industrial production of bio inoculants, respectively.

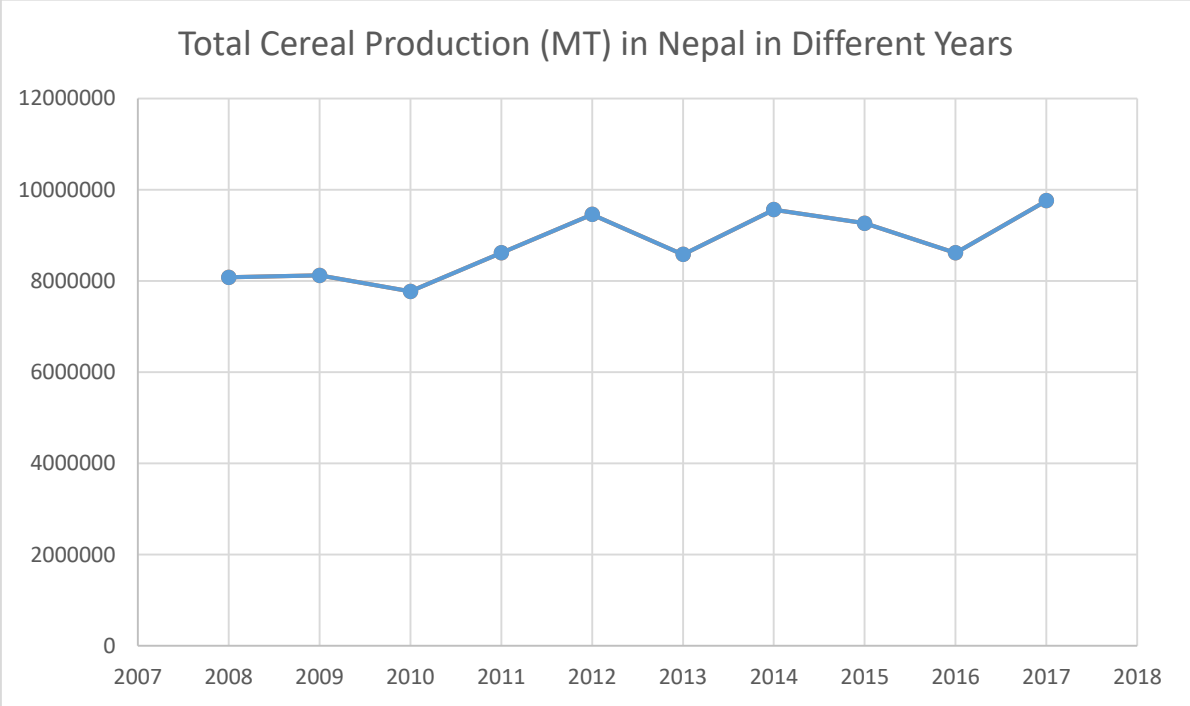
III. CURRENT PRODUCTIVITY SCENARIO OF NEPAL

Cereal crops have been a major element in addressing food security issues in Nepal. In recent years various fluctuation and different crop demand situation have been observed due to different reasons. The domestic production has not been enough to meet the rice demand in the recent years (MoAD/FAO/WFP, 2015). The five years import data of Nepal shows that the import value has inclined from NRs. 44.43 billion in 2009-10 to NRs. 127.51 billion in 2013-14 and it's still rising (Gairhe et. al, 2018).While we break the import statistics for the year in 2013-14 into parts, it is evident that of cereals from India was NRs. 35.12 billion of which rice share was NRs. 23.79 billion and the maize share was 7.43 billion (Kathmandu Post, 2015).

Timsina et al (2012) studied the status of food productivity and security and r reported that Nepal has sufficient food to meet the national demand which is in contradiction to the import statistics. The terai region of Nepal had about 506247 t of food surplus in 2011, though the remaining two geographical regionsi.e Hills and Himalayas were facing the problem of food deficiency (Gairhe et. al 2018, Timsina et. al 2012). The Maize productivity in Nepal seemed to be growing by 2.4 % between the year of 2001 and 2009(IFPRI, 2011; Gairhe et. al 2018).

Table1: Total Cereal Production in Nepal in Different Years

SN	Year	Value (MT)	Change in percent
1	2017	9758893	13.29
2	2016	8614283	-7.04
3	2015	9266240	-3.10
4	2014	9562680	11.45
5	2013	8580285	-9.28
6	2012	9457722	9.78
7	2011	8615383	10.87
8	2010	7770976	-4.33
9	2009	8122431	0.56
10	2008	8077057	10.09



(World Data Atlas, 2017)

Shrestha et. al, (2011) studied the productivity scenario of pulse grain in Nepal and found that the share of Nepal in global pulse production was 0.4%. The pulses like soybean is mainly grown in Nepal which occupies nearly about 10% of total cultivated land and ranks fourth in area after rice, wheat and maize (Shrestha et. al, 2011). Similarly, Grain legumes are grown in an area of 319,472 ha with production and productivity of 262,357 and 821 kg/ha, respectively (MoAC, 2009).

Area and production of fruits growing in Nepal is increasing as compared to previous year. According to the report published by the of Agricultural Development, Nepal, the total contribution of the various horticultural fruits i.e. orange, mango, banana and apple in AGDP is 1.4 percent, 3.88percent, 0.99percent and 0.44percent respectively. The

total area covered for apple cultivation is 10926 ha and the production are 48946 MT and productivity is 8.63 MT/ha. Similarly, the fruits like banana and mango covers an area of 12503 ha and 40110 ha respectively. Likewise, the production of banana is 168484 MT and the production of mango is 328883 MT whereas banana and mango gives productivity of 15.23 MT/ha and 10.21 MT/ha respectively (Devkota, 2017, MoAD, 2013).

IV. GENDER ROLES AND WOMEN PRODUCTIVITY

In industrialized countries, less developed and developing countries, gaps in earnings by gender have long been evident. For the equivalent kind of work, women wages are found to be 60-75% of men wages (Ali and Shields, 2010). The reason behind existence of this gap could be the women being less productive in certain works or the women being paid less than the marginal revenue product (Kumar and Hotchkiss, 1998). Nepalese industry absorbs only 6% of the labour work force (Cooke, 2000). A majority of women are dependent upon agriculture than i.e. 91% of employed women are dependent upon agriculture than 64% of the males. (Ali and Shield, 2010). The high dependence of women on agriculture makes agricultural productivity a factor of concern. Nepal has an agriculture-based economy where women participate in almost all the activities of the agriculture as done by males. Gender based discrimination has got severe against women and their earnings and productivity. Discrimination against women can be noticed by simply looking at the wage rates. Coppin, (1998) also looked at wage differentials for agricultural laborers, and found that economic development and the adoption of new technology narrows the male/female wages. Thus, women productivity in Nepal is pretty low and is dependent upon multiple factors. For the improved agricultural productivity, moth male and women should be treated equally in all the aspects including wages and opportunities. Both male and female has equal roles for agriculture enhancement.

V. STATUS OF WOMEN INVOLVED IN AGRICULTURE SECTOR OF NEPAL

Agriculture is considered as the backbone of Nepalese economy. In Nepal, more than 68% of active population are involved in agriculture in which the maximum participation in agriculture activities are carried out by women, especially by residing in rural areas (USAID, 2017). Moreover, agriculture sector contributes to about 28% of national GDP in Nepal while the average GDP from agriculture of many countries is 10 %(NRB,2018; USAID, 2017). Keeping in account to this context, the agriculture sector can further be enhanced by empowering women into agriculture.

Agriculture is a sector which is mainly comprised of production, processing and marketing. But in the context of Nepal, nearly about 80% of the women are involved in agricultural production sector as the labour forces than in processing and marketing dimensions (Sharma, 2018). The main reason why the women are lagging behind in other prospects of agriculture like industrial processing and marketing could be illiteracy and lack of ownership of agricultural resources such as land and agricultural equipment. (Sharma, 2018). Similarly, social structure and lack of exposure of scientifically advanced technologies can be other reason of poor status of women in Nepal.

VI. FOOD CRISIS AND GENDER INEQUALITY

Food security is a complex issue, which deals with adequate food availability, economic and physical access to what is available. The food quality and its micro nutrient parameter is of equal importance. The unavailability of the quality food to the consumers lead to food crisis. The unavailability may be resulted due to lower production, higher production losses, higher population, and unequal participation of the population in agriculture. The role of women is pivotal in each of the cases. Gender equality is considered as one of the most essential components of sustainable agriculture, food security and poverty alleviation. Equitable access to more and better jobs in rural areas enable rural women to become effective economic actors and engines of growth; aid them in uplifting their standard of living by improving better quality of life (FAO,2010).

There's no debate about the importance of women for rural economic growth, poverty reduction and mitigating food crisis issues. Women are significant contributors to food availability as they are the major food producers.

Rural women have the ability to lift their households and communities out of poverty. But the persistent gender inequities are hampering women involvement which is in terms limiting their access to decent work. Hence, due to the unequal involvement of the population in agriculture all the aspects of it like productivity, processing and marketing gets affected which has given arises to a crucial situation of food crisis.

VII. IMPACTS OF GENDER INEQUALITY IN AGRICULTURE SYSTEMS

The participation of smallholder farmers is very critical for the agricultural development (Bill and Melinda foundation, 2008). The agricultural produce must be brought to the market for gaining the economic advantages. One of the constraints in guiding the agricultural commodities from farm to the market is Gender. However, ability for developing countries to perceive women as agents of food and nutritional security is influenced by a variety of factors. Women are the major agricultural producers and also are key agents of food and nutritional security (World Bank Group. 2015). But in the developing countries like Nepal they have less access to productive assets such as land and services such as finance and extension, relative to men. The ability of women to meaningfully participate in collective action as members of agricultural cooperative or water user associations is also guided by a number of factors in Nepal (Aguirre and Pietropaoli, 2008). Gender in a key element in agriculture because for better nutrition cannot be implemented and succeeded without taking into account of the social, economic and biological differences between men and women (Mtsor and Idisi, 2014). In spite of the substantial importance Nepal's extension systems do not sufficiently address the needs of female farmers or rural workers. So, from the above scenario it can be generalized that gender inequality results in less productivity which eventually causes less food to grow and distribute. Lesser productivity leads to lesser income and hence causes higher risk to poverty and food insecurity. Agriculture in Nepal if done properly with the adequate utilization of scientific tools and technologies along with the equal participation of both the men and female population could lead to poverty alleviation and improved life standards and quality of life. Hence, for the agricultural growth, gender disparities must be addressed and effectively reduced (Ogunlela and Mukhtur, 2009).

VIII. METHODS OF ENSURING SUSTAINABILITY IN AGRICULTURE

Simply, Sustainable agriculture means sustainable production system based on natural processes in which maximal but sustainable use of local resources is focused. Sustainable agriculture refers tothe form of agriculture which aims to meet the needs of present generation without jeopardizing the resource base of future generation. A sustainable system will possess the features like ecologically sound, economically viable, socially justifiable, humane and adaptable (Velten et.al,2015). Species diversity is essential to achieve self-regulation and resultant stability which is maintained through biological processes. Local resources must be used in a way that minimizes losses of nutrients, biomass, and energy and avoids pollution. More emphasis is given on the use of renewable resources. Economic viability must be there which means farmers can produce enough for self-sufficiency and/or income and gain sufficient returns to warrant the labour costs involved. Equal opportunity to participate in decision making, in the field and the society must be taken in consideration (FundsforNGOs,2016). The resources and the power must be distributed in such a way that basic needs of all the members should be met. The needs like rights to land use, adequate capital, technical assistance, market opportunities must be assured. The fundamental dignity of all human beings must be recognized, and relationship and institutions incorporate such human values as trust, honest, self-respect, co-operation and compassion. For flourishing agriculture, strong communities and vibrant culture is required. There must be the behavior of adaption by the rural communities in such way that they must be capable of adjusting to the constantly changing conditions for farming, viz. population growth, policies, market demand etc. This not only involves the development of new, appropriate technologies but also innovations in social and cultural terms.

Fig.1: *Characteristics of Sustainable Agriculture*

Tey et.al (2012)

IX. CONCLUSION

The changing food consumption patterns, population growth and reduced crop productivity has resulted in increasing food demand. The main concern of the food and agricultural sector is to provide enough quality food so as to meet the nutritional needs of a growing population in an environmentally, economically and socio-culturally sustainable way. Food production and its availability is very essential for mitigating hunger. However, food production in sufficient quantity only cannot guarantee food and nutritional security. In fact, food and nutrition security relies on four pillars: availability, access, utilization and stability. The current productivity of Nepal is enough for feeding its population. Ensuring food security is both the most basic of development issues and among the most important ones. Gender inequality is one of the leading causes of this problem and reducing those inequalities will be a critical part of the solution. The potential productivity of agriculture is reduced due to inequalities faced by women as producers and hence, overall food availability in countries also gets reduced. The agricultural sector of Nepal contributes 28 percent to national GDP and this share tends to rise in the future years. At present the productivity of Nepal is sufficient to feed its population but there are a lot of problems related to gender inequality which eventually affects food and nutritional security. Such issues should be addressed with the sustainable approaches and immediate action and care is required for the mitigation of the problems.

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Karamunting (*Rhodomyrtus tomentosa*) Callus Induction *In Vitro*

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Abstract**—** *Karamunting plant is one of the biodiversity that must be developed because it has potential as phytopharmaca. The lack of public attention to the preservation and conservation of karamunting plants causes the scarcity of these plants, so it is necessary to do conservation in the form of propagation in vitro. One of the first steps that can be done is to get a callus induction protocol of karamunting plants. This study aim is to obtain a callus induction protocol for karamunting plants using 2,4D, TDZ and BAP growth regulators in vitro. This research was conducted in October to December 2019, at the Tissue Culture Laboratory of the Faculty of Agriculture, Andalas University, Padang. This experiment was compiled based on a Completely Randomized Design (CRD).The treatment used was MS media with a combination of 2,4D, BAP, and Thidiazuron concentrations, namely: A = 2.5 ppm 2,4D, B = 5.0 ppm 2,4D, C = 2.5 ppm 2,4D + 1 ppm BAP, D = 5.0 ppm 2,2D + 1 ppm BAP, E = 2.5 ppm 2,4D + 2 ppm TDZ, F = 5.0 ppm 2,4D + 2 ppm TDZ. Explants in the form of karamunting leaves from seed germination in vitro. Based on the results of the study found the influence of growth regulators BAP, 2.4D and TDZ on the percentage of explants forming callus, callus texture and karamunting callus color. By administering 2.5 ppm 2,4D + 1 ppm BAP, 5.0 ppm 2,2D + 1 ppm BAP and 2.5 ppm 2,4D + 2 ppm TDZ are able to produce a 100% callus percentage. The 5.0 ppm 2,4D + 2 ppm TDZ treatment produced crumb callus with the highest percentage which was 90%, and 5.0 ppm 2,4D was able to produce compact callus with the highest percentage which was 100% and the 2.5 ppm 2,4D treatment, 2.5 ppm 2,4D and 5.0 ppm 2,4D + 2 ppm TDZ produce white callus with the highest percentage that is 100% and 2.5 ppm 2,4D treatment + 1 ppm BAP produces green callus with the most percentage which is 75%. While for the first time the callus appeared there was no effect of some concentrations of BAP, TDZ and 2,4D.***

Keywords **— callus, conservation, induction, karamunting, phytopharmaca,**

I. INTRODUCTION

Karamunting (*Rhodomyrtus tomentosa*) is a plant that has the potential as a biopharmaca plant because it has been proven to have medicinal properties in every part of the plant. Several compounds in karamunting such as flavonoids as antibacterial and antioxidant, saponin as an antiseptic, tannin as an astrigent that is able to cover the skin pores and light bledding [1] Karamunting root can be beneficial to increase platelet count, fibrinogen level, and contractile muscle of smooth blood vessels. Caramunting fruit can increase the level of hemoglobin and the number of red blood cells (cause adaptive effects), increase antianoxic, cold and fatigue resistance[2]. Some alkaloid compounds are efficacious as anti-diarrhea, anti-diabetic, anti-microbial and anti-malaria, and contain flavonoid compounds to accelerate wound healing by slowing the onset of cell necrosis, increasing the strength of collagen fibers and preventing cell damage[3]. Karamunting also functions as a fever reliever (antipyretic), pain reliever (analgesic), laxative urine (diuretic), relieving swelling, blood flow and stopping bleeding (hemostasis)[4]

In recent years karamunting began to be difficult to find, this happened because there was no community effort to preserve and cultivate it. Other things that can cause these plants to become scarce are land clearing, forest burning and land conversion. So that scarcity of karamunting plants does not continue, it is important to do conservation, one of the conservation steps that can be done is propagation or conservation (short-term storage) in vitro.

In vitro conservation method is one of the first steps in conservation activities, namely by finding the right

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media for the growth of karamunting in vitro. Conservation is carried out in an effort to manage natural resources wisely based on conservation. Conservation of genetic resources needs to be done in order to preserve and preserve the existence of karamunting. Conservation in vitro is divided into short-term, medium-term and long-term (cryopreservation) conservation. Short-term conservation technique or often called the optimal growth technique is a technique of storing planting material in the media and optimum conditions so that explants can grow optimally. Propagation of plants with tissue culture techniques has been done for plants of high economic value or plants that are classified as rare and difficult to propagate conventionally.

Callus induction is one method of tissue culture that is done by stimulating cell division continuously from certain plant parts such as leaves, roots, stems, and so on by using growth regulators to form cell mass. The cell mass (callus) will then regenerate through organogenesis or embryogenesis to become a new plant. In guava callus induction, giving 2,4D growth regulators with a concentration of 5 ppm and 5 ppm 2,4-D + 1 ppm BAP is the best treatment to induce guava callus at 1-2 MST, then by giving 2.5 ppm 2,4-D + 2 ppm 2-Ip is the best treatment in inducing compact callus at 4 MST[5]. Then the results of by administering 2,4-D growth regulators with TDZ gives an influence on the formation of callus in jatropha (*Jatropha curcas* L.) plants, where at a concentration of 5 ppm 2,4-D + 1 ppm TDZ can produce the fastest callus emergence time, whereas by giving 2.5 ppm 2,4-D + 1 ppm TDZ produces the percentage of formation highest callus in jatropha plants[6]

The purpose of this study was to obtain a callus induction protocol for karamunting plants using 2,4D, TDZ and BAP growth regulators in vitro.

II. MATERIAL ANDMETHOD

2.1 Implementation Research

This research was conducted in October to December 2019, at the Tissue Culture Laboratory of the Faculty of Agriculture, Andalas University, Padang. The tools used in this study are laminar air flow cabinet, hot plate, magnetic stirrer, scales, measuring flask of various sizes, pasteur pipettes, ovens, erlenmeyers, goblets, stirring glasses, culture bottles, test tubes, petri dishes, spatulas, scissors, scissors rubber band, aluminum foil, scalpel, tweezers, autoclave, bunsen, hand spayer, pH paper, label paper, camera, stationery, and clear plastic.

The materials used are nodes and karamunting leaves obtained from seed germination in vitro, MS media, sucrose, bacto agar, pH regulating solution, 70% alcohol, 96% alcohol, distilled water, bayclin (bleach containing the active ingredient sodium hypochlorite 5 , 25%), spritus, growth regulators BAP, 2,4D, and Thidiazuron, detergents, duct tape, label paper, aluminum foil, and HVS paper.

This experiment was compiled based on a Completely Randomized Design (CRD). The treatments used were MS media with a combination of 2,4D, BAP, and Thidiazuron concentrations, namely:

A = 2.5 ppm 2,4D

B = 5.0 ppm 2,4D

C = 2.5 ppm 2,4D + 1 ppm BAP

D = 5.0 ppm 2,2D + 1 ppm BAP

E = 2.5 ppm 2,4D + 2 ppm TDZ

F = 5.0 ppm 2,4D + 2 ppm TDZ

Thus there were 6 treatments, each treatment was repeated 5 times so that there were 30 experimental units. Each experimental unit consisted of 2 bottles so that the number of bottles used was 60 bottles. In each culture bottle 2 explants were planted and all were observed. The observational data were analyzed using the F test and when significantly different it was followed by DMRT at the 5% level.

Tools such as petridish, scalpel, culture bottles, tweezers, and other equipment are washed with detergent and rinsed thoroughly, then the bottle is immersed in 20% bayclin for 24 hours, then sterilized in an autoclave at a pressure of 15 Psi (pounds per square inch = pressure on an area of 1 inch) with a temperature of 121^{o}C for about 20 minutes. Tools other than culture bottles are wrapped in HVS paper and then wrapped in clear plastic before being put into the autoclave. The water in the autoclave is changed every time you use it. The tools used after sterilization are stored in the oven until used. Laminar air flow is sterilized using UV light for 1 hour before planting and sprayed with 70% alcohol each time it will be used and after use.

2.2 Media Making

The media used is MS media. The composition of MS media can be seen in Appendix 3. How to make MS media with a volume of 1 L is inserted 800 ml of sterile aquadest into a 1 L size erlenmeyer, then put a solution of macro stock, micro stock, iron stock, Mg stock, and vitamins according to raw requirements for making MS media. 30 g / L sucrose was added, and myo-inositol 100 mg / L, then sufficient media volume was approached to 1 liter. Furthermore, the treatment media solution was divided

into 6 at 250 ml size erlenmeyer. After that added growth regulators 2,4D, BAP and TDZ according to each treatment, then the pH of the solution was measured with pH paper, the pH of the solution ranged from 5.6 to 5.8 and if the pH of the solution was less than 5.6 then added NaOH 0 , 1 N and if the pH of the solution is more than 5.8, 0.1 HCl of HCl is added. Then bacto is added so that 8 g / L which has previously been divided by six and added to each treatment. Finally the media is heated on a hot plate until it boils. After the media boils the solution is poured into a culture bottle as much as 20 ml / bottle. Then the bottle is closed using glass plastic and tied with a rubber band. After that, the media was sterilized in an autoclave at 121 ° C, a pressure of 15 Psi for 15 minutes. The media is stored in a culture rack before planting.

2.3 Preparation of explants

In the preparation of explants using explants as a result of in vitro karamunting germination using leaf explants (Figure 1). Seed germination was carried out on MS + 1 ppm GA3 media. Sterile leaf explants from karamunting germination in vitro can be seen in Figure 2.

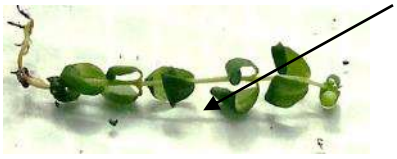

Fig.2. Leaf sterile explants used as a result of karamunting germination in vitro

2.5 Planting

Young leaves are cut at the tip and base of the leaf so that the tip and base of the leaf is the injured part of the leaf. Leaf explants were put into culture bottles containing treatment media using tweezers. Then the bottle is closed using a sterile plastic before and tied with rubber. Planting is carried out in a Laminar Air Flow Cabinet (LAFC) which has been previously exposed to UV for 1 hour and is equipped with the tools and materials needed to be sprayed first with 70% alcohol before being forced into LAFC. Culture bottles are arranged on a culture rack with a temperature of ± 25 ° C.

2.6 Observation

2.6.1 Soft start time (HST)

Observation Aiming for the first day of explants forming callus from each treatment combination. Observations were made by observing the development of explants starting from the day of planting until the formation of callus formed from leaf injury.

2.6.2Percentage of Forms Forming Callus (%)

This observation aims to see the ability of explants to form callus. Observations were made from the first week to the day when there were no more explants forming callus.

$$\% \text{ Eksplan forming callus} = \frac{\sum \text{explants forming callus}}{\sum \text{planted explants}} \times 100\%$$

2.6.3 Callus Texture

Callus texture was visually observed, the part observed was the outer shape of the callus by observing each sample from outside the culture bottle. The observations were documented using a digital camera and explained descriptively.

2.6.4 Callus Color

Observation of color is done by observing the change in color of the callus. Observation of callus color was done by comparing callus with Muncle color chart paper for plant tissue, documented using a digital camera and explained descriptively.

III. RESULTS ANDDISCUSSION

3.1 Callus Time (HST)

The results of the analysis of variance showed that the combination of several concentrations of 2,4-D, BAP and TDZ had no effect on the day of callus appearing on caramunting leaf explants. When the karamunting call appears, it can be seen in Table 1.

Table 1. Time of callus emergence in caramunting leaf explants by treating several concentrations of 2,4D, BAP and TDZ

Treatment	Callus Time (HST)
2,5 ppm 2,4D	18.55
5,0 ppm 2,4D	17.70
2,5 ppm 2,4D + 1 ppm BAP	19.65
5,0 ppm 2,4D + 1 ppm BAP	19.70
2,5 ppm 2,4D + 2 ppm TDZ	20.37
5,0 ppm 2,4D + 2 ppm TDZ	21.00

Data differ significantly based on F test of 5% level

The observations in table 1 show that callus in karamunting leaf explants appeared on days 17.7-21.0 days after planting. Karamunting callus induction callus is relatively long compared to plants with the same family. Guava plants need about 7 DAP to induce callus[5].Callus induction in wheat plants was relatively fast at around 4 HST using explant of young embryos[7]

ZPT of the auxin and cytokinin group given is able to trigger the division so that the process of callus formation can work. In addition, the emergence of the callus is

thought to be due to increased endogenous auxin content in the leaves due to the addition of exogenous auxin. Manyauxin is synthesized in the apical bud meristematic tissue and young leaves, so that in meristematic young leaves near the end of the stem, the auxin levels are high. The appearance of callus on karamunting leaves is marked by the swelling of explants in the wound site or leaf bone[8].

Callus formation occurred because of the injury given to the explant so that the cells in the explant would repair the damaged cells. The initial stage is an increase in cell wall permeability and water absorption, so that cells will swell then cell division will occur which will form a callus. Callus morphogenesis depends on the balance of auxin and cytokinin in the media[9]. The interaction between plant endogenous growth regulators and exogenous growth regulators absorbed in the media will determine the direction of callus development [10].Callus formed more quickly when auxin concentrations increased. Callus usually appears first in the injured explant area. The presence of injury in explants makes it easier for exogenous ZPT to diffuse into the tissues and works together with endogenous ZPT to form callus by stimulating cell division, especially in the wound area.[11]. The emergence of callus on the injured part is also due to the stimulation of explant tissue to cover the wound that begins with cell wall expansion and water absorption, then the cell will carry out cell division[12].

3.2 Percentage of Forms Forming Callus (%)

The results of the analysis of variance showed that the combination of several concentrations of 2,4D, BAP and TDZ had an influence on the percentage of callus formation in karamunting explants. The percentage of karamunting callus formation can be seen in Table 2.

Table 2. Percentage of Callus Formation in Karamunting Explants with Treatment of Several Concentrations of 2,4D, BAP and TDZ

Treatment	*Percentage of Callus* (%)
2,5 ppm 2,4D	45 b
5,0 ppm 2,4D	30 b
2,5 ppm 2,4D + 1 ppm BAP	100 a
5,0 ppm 2,2D + 1 ppm BAP	100 a
2,5 ppm 2,4D + 2 ppm TDZ	100 a
5,0 ppm 2,4D + 2 ppm TDZ	55 b

Figures followed by the same lowercase indicate significantly different based on the DMRT test at 5% level

Treatment of 2.5 ppm 2,4D + 1 ppm BAP, 5.0 ppm 2,2D + 1 ppm BAP and 2.5 ppm 2,4D + 2 ppm TDZ were able to produce the highest percentage of callus that is 100% and 5.0 ppm treatment 2,4D produces the lowest callus percentage of 30%. This shows that 2.4 D growth regulators work more optimally when combined with cytokines such as BAP and TDZ in the formation of callus in karamunting explants.

The use of different ZPT gives a variety of responses to each explant individual. This is due to the interaction between endogenous hormones in plant tissue and given exogenous growth regulators[13].Addition of 2.4 D in the media will stimulate cell division and enlargement in the explants so as to stimulate callus formation[14]. The addition of BAP into the media was able to play an active role in the growth and ploriferation of callus[15]. Growth regulating substances which are very influential in the formation of callus are auxin and cytokines, in this case 2,4D with BAP and Kinetin which are able to increase endogenous ZPT content in cells so that they are able to trigger growth and tissue development.. Very active growth substances which influence the formation of callus are auxin and cytokinin, in this case 2,4D with BAP and Kinetin which are able to increase endogenous ZPT content in cells so that they are able to act as a trigger for tissue growth and development[16]

Formation of regenerable callus in plants generally depends on genotype, source of explants, physiological conditions of donor plants, tissue type, media, type and concentration of PGR, and the interaction of these factors [17] The use of auxin is able to activate the transduction signal so that cells can reorganize gene expression and induce cell division to callus growth and somatic embryogenesis. The interaction between endogenous and exogenous ZPT will determine the direction of culture development. The addition of auxin to the medium will change the endogenous ZPT ratio which then becomes a determining factor for the growth process and morphogenesis of explants. Embryogenic callus induction generally uses media with auxin content which has strong activity[18].

3.3 Callus Texture

Callus is a disorganized collection of cells and occurs because of a very active division. Stimulation of endogenous hormones or growth regulators that are added (exogenous) causes cell metabolism to become active, in such circumstances the tissue is said to be undergoing de-differentiation. This situation continues during callus proliferation[19]. Callus that has a crumb texture grows

apart into small pieces, has a lot of water content, easily separated [20]. Callus texture of caramunting leaf explants can be seen in Table 3.

Table 3. Callus texture of karamunting plants at 2,4D, BAP and TDZ concentrations

Treatment	*Callus texture* (%)	
	Crumb	Compact
2,5 ppm 2,4D	20 cd	80 a
5,0 ppm 2,4D	0 d	100 a
2,5 ppm 2,4D + 1 ppm BAP	75 ab	25 c
5,0 ppm 2,2D + 1 ppm BAP	45 bc	55 b
2,5 ppm 2,4D + 2 ppm TDZ	80 a	20 c
5,0 ppm 2,4D + 2 ppm TDZ	90 a	10 c

The number followed by the same lowercase letter in the column shows no significant difference based on the DMRT test at 5% level

The results showed that with some 2,4D treatment, BAP and TDZ were able to produce crumb and compact callus. The 5.0 ppm 2,4D + 2 ppm TDZ treatment produced crumb callus with the highest percentage which was 90%, and 5.0 ppm 2,4D was able to produce compact callus with the highest percentage which was 100%. The combination of 2,4-D 0.3 mg / l + BA 0.1 mg / l is the best treatment that can produce a more crumb callus texture in plants Dutch teak[21]. Callus texture is a marker of the quality of a callus. The texture of the callus can vary from compact to weak, depending on the type of plant used, the composition of nutrient media, growth regulators and the environmental conditions of culture[23].The formation of crust structured callus is triggered by the presence of endogenous auxin hormones that are produced internally by explants that have arisen to form the callus[23]. The formation of crumb callus is also influenced by the addition of cytokinins (BAP) in media.

The formation of crust structured callus is triggered by the presence of endogenous auxin hormone which is produced internally by explants that have arisen to form the callus. The formation of crumb callus is also influenced by the addition of cytokinins (BAP) in media that already contains auxin9[23]. The presence of cytokinins can increase cell division in the cytokinesis process, in addition ZPT acts as a trigger especially when RNA and protein synthesis will increase cell division to form callus[21]. The formation of crumb callus is the result of increased cell division activity

The formation of crumb-textured callus in this study was thought to be triggered by the presence of high auxin hormone, in this case 2,4-D[22]. The addition of 2,4-D causes cells to be more actively dividing and enlarging to produce crumb callus. Visually, crumb callus is formed in explants, the cells are small and clustered, the bonds between cells appear to be tenuous, if taken with tweezers, they break easily and some are attached to the tweezers[24]. The crumb callus texture is considered good because it makes it easy to separate into single cells so that the effort to multiply in terms of the number of callus will be easier[25].

In addition to producing crumb callus, karamunting explants also produce a compact callus. Compact callus has cells that look elongated and overlap together, from callus assemblages, and have the potential to develop toward organogenesis and not potentially become somatic embryos[26]. Compact callus which has a dense tissue arrangement, contains a lot of water, greenish color and has meristemoid tissue is very supportive for micropopagation that produces plantlets and for shoot culture purposes[27]. Callus texture produced by the combination treatment of several growth regulators 2,4D, TDZ and BAP can be seen in Figure 2

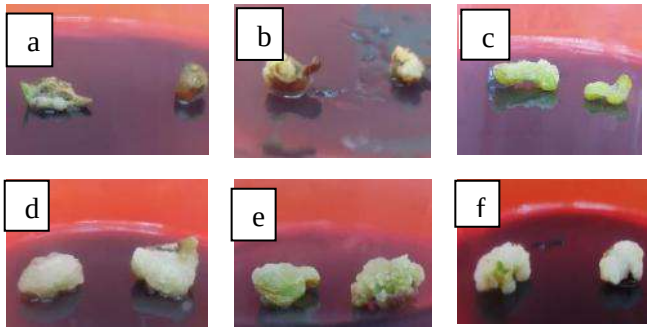

Fig 2. Crumb callus texture produced at several concentrations of growth regulator 2,4D, TDZ and BAP (a) Crumb textured callus at 2.5 ppm 2,4D (b) Callus at 5.0 ppm 2,4D (c) Callus at a treatment of 2.5 ppm 2,4D + 1 ppm BAP (d) Callus at a treatment of 5.0 ppm 2.2D + 1 ppm BAP (e) Callus at treatment 2.5 ppm 2,4D + 2 ppm TDZ and (F) Callus at treatment 5.0 ppm 2,4D + 2 ppm TDZ

3.4 Callus Color

The indicator of explant development in in vitro culture in the form of callus color and texture is used to describe the visual appearance of the callus so that it can be seen that the cell is still actively dividing or has died. Karamunting callus explant color with several treatments of ZPT 2,4D. BAP and TDZ can be seen in Table 4.

Table 4. Color of Karamunting Callus with several treatments of growth regulator 2,4D, BAP and TDZ

Treatment	Color of Callus (%)	
	White	Green
2,5 ppm 2,4D	100 a	0 b
5,0 ppm 2,4D	100 a	0 b
2,5 ppm 2,4D + 1 ppm BAP	25 b	75 a
5,0 ppm 2,2D + 1 ppm BAP	85 a	15 b
2,5 ppm 2,4D + 2 ppm TDZ	80 a	20 b
5,0 ppm 2,4D + 2 ppm TDZ	100 a	0 b

Figures followed by the same lowercase indicate no significant difference based on the DMRT test at 5% level

Table 4 shows that explants in karamunting leaves are capable of producing white and green callus. In the treatment of 2.5 ppm 2,4D, 2.5 ppm 2,4D and 5.0 ppm 2,4D + 2 ppm TDZ produced the highest white callus with the highest percentage of 100% and 2.5 ppm 2,4D + treatment 1 ppm BAP produces the highest green callus with a percentage of 75%. The color of the callus is also an indicator of callus growth. The white callus is embryogenic tissue that does not yet contain chloroplasts, but has a high starch content [28]. The callus color identifies the presence of chlorophyll in the tissue, the more green the callus color, the more chlorophyll content in the callus [29]. The difference in color of the callus shows the level of development of the callus. Callus color indicates the presence of chlorophyll in tissues, where the more green the callus color, the more chlorophyll content it has and the white color in the callus indicates the callus condition is still quite good[24]. The green color in callus is a result or effect of cytokinin in the formation of chlorophyll. Plant growth regulators also have a role in the color of the callus[23]. Increasing concentrations of cytokinins are increasingly likely to show a bright green color on a more durable callus. This is related to the role of cytokinins which can slow the process of senesensi (aging) of cells by inhibiting the overhaul of the grains of chlorophyll and protein in cells[24].

IV. CONCLUSIONS

Based on the results of the study concluded that the influence of growth regulators BAP, 2.4D and TDZ on the percentage of explants formed callus, callus texture and color karamunting callus. By giving 2.5 ppm 2,4D + 1 ppm BAP, 5.0 ppm 2,2D + 1 ppm BAP and 2.5 ppm 2,4D + 2 ppm TDZ are able to produce a 100% callus percentage. The 5.0 ppm 2,4D + 2 ppm TDZ treatment produced crumb callus with the highest percentage which was 90%, and 5.0 ppm 2,4D was able to produce compact callus with the highest percentage which was 100% and the 2.5 ppm 2,4D treatment, 2.5 ppm 2,4D and 5.0 ppm 2,4D + 2 ppm TDZ produce white callus with the highest percentage that is 100% and 2.5 ppm 2,4D treatment + 1 ppm BAP produces green callus with the most percentage which is 75%. While for the first time the callus appeared there was no effect of some concentrations of BAP, TDZ and 2,4D.

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Analysis of Economic Benefits in Reclamation Activities and Coastal Conversion in Barru District

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Abstract*— *This research was conducted in Barru District, South Sulawesi. The purpose of this study is to analyze how much the total economic value of coastal land ecosystems in Barru District. Analyzing the value of economic benefits and the multiplier effects of coastal ecosystem reclamation activities in Barru District. Analysis of the data used is to use economic valuation analysis by calculating the value of direct benefits, indirect benefits, the value of option benefits, the value of the benefits of existence, and the value of bequeathing benefits. The results showed the total economic value in the Garongkong hamlet was Rp4,114,881,797 with an average value of Rp2,839,099,322, whereas for Lawallu village had a total economic value of Rp863,980,557 with an average value of Rp39,776,590. From these results, it can be seen that reclamation can provide a higher economic value compared to land conversion. With the reclamation, it can create opportunities for economic improvement of the community around the reclamation land.

Keywords*— *Mangrove Ecosystem, Value of Economic Benefits, Reclamation, Coastal area of Barru District.

I. PRELIMINARY

Indonesia is a country with the largest area of waters in the world, 75% of Indonesia's territory are oceans and the oceans have enormous economic potential for the development of the life of the Indonesian people and the improvement of Indonesia's welfare in general. The development of maritime territory for the last three decades has always been positioned as a periphery in national economic development. This condition is ironic considering that Indonesia's territory is very strategic in an important geo-political position namely the Pacific Ocean and the Indian Ocean where this region is the most dynamic region in the world political-economic arena. This makes Indonesia's marine potential should be the foundation of economic development.

Coastal is an area and ecosystem that is rich and potential to be used for various activities. The great potential becomes very important as the initial capital of development. Development is then directed at improving the people's welfare and social justice. On the other hand development must also continue to maintain the sustainability of the development itself, without damaging the functioning of the environmental ecosystem. Efforts to meet the needs and creation of an advanced economy on the one hand are very important, bearing in mind that macro economic progress is expected to encourage the creation of a prosperous society and ultimately a prosperous state. On the other hand economic development often ignores the functions of the surrounding environment. This condition causes damage to the ecosystem and leads to loss of resources (scarcity resources).

Barru District is located in the southern peninsula of Sulawesi Island, which is administratively part of the South Sulawesi Province, the area extending from North to South directly facing the Makassar Strait. Practically along the ± 72 km West side or about 22.4% of the total area of this district is a coastal area, with an altitude of 0-25 m above sea level (Anonymous, 2013; 2010). With such a geospatial position and configuration, the coastal and marine areas are the basis of natural resources which are the main components forming the basic character of society as well as socio-cultural, typological relations between regions and economic characteristics of the region.

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In 2013, a port was announced officially in Barru sub-district, Barru District, South Sulawesi. The port has been built since 2015 by conducting reclamation activities. Reclamation is one of the potential solutions to increase demand for new land for living and development. In the past, many coastal countries, such as the Netherlands (Hoeksema, 2007), United Kingdom (OSPAR Commission, 2008), Japan (Suzuki, 2003), South Korea (Children and Wang, 2009) and Singapore (Glaser et al., 1991), have exploited Covers and reclaims the sea extensively (henceforth abbreviated as land reclamation) for the expansion of coastal cities, for land for industry and agricultural development along the coast, and also for defense against storm surges.

There are three objectives of the reclamation program are (Wagiu, 2011):

a) To get back the land lost due to the sea waves
b) To acquire new land in the front line of the coastline to erect a building that will function as a shoreline protection fortress
c) For economic reasons, construction or to erect large-scale building construction.

Conversion and the use of mangrove forests by cutting down forests and transferring their functions to other uses will have a very broad impact. Harvesting of forest products and conversion of mangrove forests can provide results to community income and opportunities to increase employment. But on the other hand, there was a decline in mangrove forests, which in turn could disrupt the surrounding aquatic ecosystem.

II. METHOD

Research activities on the reclamation and conversion of coastal land were performed in Barru District, taking two sub-districts of Barru District as research samples. This study took the location of reclamation and land conversion. For the reclamation area, it is located in the Garongkong Sea Port, precisely in the Garongkong neighborhood, Mangempang Village, Barru District, Barru District. For land conversion, the location is only in Lawallu Village, Soppeng Riaja District, Barru District. This research was conducted for 3 months.

Data collection

Based on the research objectives, the data collected in this study consisted of two sources, namely primary data and secondary data. Primary data, i.e. data obtained directly from measurements and observations in the field. This primary data is sourced from coastal locations, fishermen or farmers, tourists, the general public, and stakeholders who have an interest in coastal ecosystems. Secondary data, namely supporting data collected from departmental, government and private agencies, as well as from publications and research results that have been carried out previously.

Economic Valuation Analysis

Generally, assessments occur based on interactions between humans as subjects (assessors) and objects (something that is valued). Each individual has a different perspective in assessing things. Every individual has a number of values that are said to be the value of mastery (head value) which is the basis of individual preferences. In the end the value of the object is determined by the various values that are stated (assigned value) by individuals (Pearce and Turner, 1994 in Djijiono 2002).

$$\mathbf{TEV = DV + IV + OV + EV + BV}$$

Remaks:

TEV = Total economic value

DV = Value of direct benefit

IV = Value of indirect benefit

OV = Value of option benefit

EV = Value of existence benefit

BV = Value of bequeathing benefit

Each of these values is identified based on all the benefits gained in the mangrove ecosystem studied. Each of these values is as follows:

a. Direct Use Value (DUV)

The formula used to get the total value of direct benefits is according to the following:

$$TML = ML1 + ML2 + ML3 + \ldots + MLn$$

Where :

TML = Total Direct Benefits

ML1 = Direct Benefits of Fish

ML2 = Direct Benefits of Crabs

ML3 = Direct Benefits of Shells

ML4 = Direct Benefits of Mangrove Wood

b. Indirect Benefits

Value of indirect benefits is the value of benefits from a resource (mangrove) that is used indirectly by the community. Indirect benefits of mangrove forests can be in the form of physical benefits, namely as a barrier to seawater abrasion. Physical assessment of mangrove forests can be estimated by the function of mangrove

forests as abrasion, feeding, spawning, and nursery ground protection. However, in this study the value of indirect benefits is only approached by using the function of mangrove forests as a barrier to abrasion. Other indirect functions require comprehensive research given the limited availability of data.

c. Option Benefits
The formula is as follows:

MP = MPPL
Where :
MP = Benefits of option
MPPL = Benefits of other usage options

d. Existence Benefits
These benefits can be formulated as follows (Ruitenbeek, 1992):

$$ME = \sum_{i=1}^{n} (MEi)/n$$

explanation:
ME = Benefits of Existence
MEi = Ecosystem benefits from the i-respondent
N = Number of respondents

e. Bequeathing Benefits
The formula is as follows :

$$ME = \sum_{i=1}^{n} (MKi)/n$$

Where :
MW = the benefits of bequeathing
Mwi = benefit of bequeathing from the i-respondent
N = total respondents

2. Analysis of WTP with CVM

According to Juanda (2009), multiple linear regression analysis (multiple regression) is a regression equation that describes the relationship between one dependent variable (dependent variable) with several independent variables. Multiple linear regression analysis in this study was used to evaluate the use of contingent valuation method (CVM). Evaluation of the implementation of the CVM model can be seen from the level of reliability of the willingness to pay (WTP) function. The multiple linear regression equation used in analyzing the factors that influence the WTP value of respondents is as follows:

WTP = β0 + β1JK + β2TP + β3PD + β4PK + β5JT...+ βnXY + εi

Where :

JK = Sex (Women and Men)

TP = Education Level

PD = Revenue

PK = Work

JT = Number of Dependents

ε = Error

I = First Respondent (i = 1,2,3, ... n)

3. Analysis of the economic benefits of conversion and reclamation

The economic impact of land conversion and reclamation is measured using the multiplier effect of the cash flows that occur. In measuring the economic impact of an activity on a community's economy, it is analyzed by dynamic modeling through the dynamics of inter-relations between vital elements along with the changing time of the ecological-economic system examined in this study. The basic concept of the formulation of the model refers to the cyclic effect where changes in the index and management attributes can affect the sustainability of the marine tourism management system. The analysis stage begins by building a causal loop diagram, then making a model base, entering the attribute values that have been obtained in the previous analysis into the model that was built, arranging the management model scenario, and finally doing a model simulation. The attribute values used in analyzing the sustainability of management are derived from the results of previous analyzes, namely land suitability analysis, area carrying capacity analysis, economic valuation of coral reef and mangrove resources, cost-benefit analysis, and literature search. Some attribute values used are obtained from scientific estimation methods, but it is recognized that the accuracy of parameter estimates depends on the availability of data from the source and the analytical method used.

Measurement of local economic impacts through several types of multiplier effects (META, 2001), namely:

Keynesian Income Multiplier is a change in tourist expenditure units giving changes to the income level of local people. Mathematically written:

$$= \frac{D + N + U}{E}$$

Ratio Income Multiplier is a multiplier effect that illustrates how much impact on the local economy. This multiplier has included further impacts and indirect impacts.

Ratio Income Multiplier Type I, mathematically written:

$$= \frac{D + N}{D}$$

Ratio Income Multiplier Type II, mathematically written:

$$= \frac{D + N + U}{D}$$

Where:

D: Local income received directly from E (rupiah)

N: Local income received indirectly from E (rupiah)

E: Additional tourist expenditure (rupiah)

U: Local income received further from E (rupiah)

The multiplier effect has the following criterias :

- If the multiplier coefficient value is less or equal to zero (≤ 0), then the reclamation and land conversion area has not been able to have an economic impact on community activities.
- If the multiplier coefficient value is between zero and one (0 ≤ x ≤ 1), then the reclamation and land conversion area has a low economic impact value.
- If the multiplier coefficient is more than or equal to one (≥ 1), the reclamation and land conversion area is able to have an economic impact on tourism activities.

III. RESULTS AND DISCUSSION

The Direct Benefits of Mangrove Resources

Barru coastal mangrove resources have a variety of benefits, both directly and indirectly. The direct benefit of mangrove resources in Barru District can be seen in the direct use of fisheries resources that exist around the mangrove forest.

The value of direct benefits is the value or benefits of mangrove forest resources obtained directly through their production and consumption. Direct benefits for the community from mangrove forests in the study area are firewood, nipa roofs, fish and crabs. Benefits are staple foods for fish, shrimp, and crabs that live in coastal ecosystems through landslides from mangrove litter (especially their leaves). A small amount of waste falls on the forest floor will be consumed by crabs and most will be decomposed by microbes that are the source of detrivora food, then detrivora becomes a source of carnivorous food (Harahap 2010; Kusmana 2010). Another benefit obtained from the mangrove ecosystem is abundance is an aquatic biota that is economically valuable. There are several types of aquatic biota around mangroves that can and are often utilized by the community including baronang fish, white grouper fish, besides that there are also tude oysters and squid.

Table 2. Total Value of Mangrove Benefits in Garongkong and Lawallu Villages

Type	Total Value of Benefits		Average Benefits	
	Garongkong	**Lawallu**	**Garongkong**	**Lawallu**
Fishes	1.241.155.366	428.261.627	33.544.739,62	26.766.351
Squids	67.540.000	-	22.513.333,33	-
Crabs	4.400.000	16.120.000	4.400.000	8.060.000
Woods	400.1400.000	400.1400.000	527.889	527.889
Total	**1.328.623.842**	**844.521.627**	**60.985.961,95**	**35.354.240**

Source : Primary Data, 2018

The measurement of direct benefits is carried out in terms of market value in order to quantify the prices of various charcoal obtained. The process of calculating the value of direct benefits of a mangrove ecosystem is done by multiplying the average net income by the total population. Net income is obtained from the selisis between the total gross income with the total costs used to obtain these resources. Total gross income derived from production is multiplied by price.

Value of Benefits of Direct Fishing

Barru District is a district in Barru District which has a height of 6 m above sea level. The total population in Barru Subdistrict is 5,791 people in 2018. Barru Subdistrict has 5 villages and 5 villages, one of which is Mangempang Village. Mangempang Urban Village has an area of 13.8 Km2 and is one of the coastal areas in Barru District. This causes some residents to choose to work as fishermen. The work is used as a livelihood in supporting increased production in the fishing sector. (BPS Kab Barru, 2019)

Soppeng Riaja sub-district in Barru District has 5 villages and 2 kelurahan, one of which is Lawallu. Lawallu Village has a population of 1,966 people. Lawallu Village has the potential for development related to businesses in the fisheries and marine sector. The characteristic of this area which is a coastal area is the dominant profession of the community earning a living as a fisherman. Nevertheless in this Lawallu village there are also several other potentials to be developed. The many mangrove forests on the coast of Lawallu Village have high economic value. (BPS Kab Barru, 2019).

Fishing is performed in the mangrove area around the Garongkong Hamlet and Lawallu Village. The type of fishing gear that is used to catch fish in the Garongkong hamlet and Lawallu Village uses the same fishing gear that is a net. The fish catches produced consist of various types of fish namely Cepak for Garongkong Hamlet,Gembong and Kakap for Lawallu Village. How to calculate the number of catches is by hanging (per-hanging), not using per kg in one time catches.

For Garongkong Hamlet, the fishes caught are Cepak. The average number of catches of Cepak is 845 hanging / year. The average selling price of Cepak from fishermen to collectors is Rp.47,027 / hanging. The number of fishermen who catch in the mangrove area around Garongkong are 37 people.

The total costs incurred in the catching of Cepak Fish business are Rp231,359,634 / year, including an investment cost of Rp436,510,000 / year. Based on the amount of production, the selling price of fish and the costs incurred for catching, the net benefit value from catching fish will be obtained in the amount of Rp1,241,155,366 / year.

For Lawallu Village, the fishes caught are Kakap and Gembong. The average number of fish caught is 744 hanging / year. The average selling price for Kakap and Gembong is IDR 45,625 / hanging. In the calculation of fish catches for the village of Lawallu combined because there is no difference in the selling price of fish to collectors. The number of fishermen who catch in the mangrove forest area around the land conversion area are 16 people.

The total costs incurred in the fishing business in the hamlet of Lawallu is Rp.115,248,373 / year, with investment costs in the fishing business amounting to Rp138,171,000 / year. Based on the amount of production, sales price and cost of catching, the net benefit value from catching Kakap and Gembong fishing is Rp. 428,261,627 / year.

The frequency of catching for Garongkong sub-village is an average of 247 fish catches a year and for Lawallu Village is around 232 times a year. Catching is done once a day to catch fish. The number of catches obtained in a year for Garongkong Hamlet is an average of 845 catches per year, while for Lawallu Village which is an average of 744 catches. For the selling price, for Garongkong hamlet, the average selling price of fish is IDR 47,027 / stick and for Lawallu Village which is around IDR 45,625. Based on this value, the total economic value of fish obtained by Garongkong Hamlet is in the amount of Rp1,241,155,366 / year and for Lawallu Village in the amount of IDR428,261,627 / year.

The Direct Benefits of Squid

Catching squids is performed by fishermen in the Garongkong hamlet using fishing nets. Theydon't consider catching squid as priority because squids are not available every day. There are 4 cumu-squid catchers in Garongkong village. Costs incurred to catch squid is Rp13,486,000 / year.

The catch a day reaches an average of 3 hanging per day and 2,228 hanging / year with a selling price of Rp 50,000 / hanging. Squid will be sold to collectors or sold directly to the market. The net catch of squid in a year is Rp100,914,000 / year.

Value of Direct Benefits of Crab Catching

The types of crabs caught by the community in the mangrove forest area in Garongkong and Lawallu villages are mangrove crabs. Mangrove crabs have habitats and live in holes and thickets of mangrove trees, while crab crabs live in coastal areas around mangrove forests.

The fishing gear used by crab catchers in the mangrove area of Garongkong and Lawallu Village uses the same tool, which is a trap made of bamboo or rattan. The number of fishermen who caught crabs in Garongkong sub-village was 1 person and in Lawallu village was 2 people. The total cost used for crab fishing in Garongkong is IDR 6,633,000 / year. For Lawallu Village, the cost of arrest is Rp14,909,835 / year.

Based on the results of interviews with respondents from each region that in one year, fishermen catching mangrove crabs in Garongkong area were performed as many as 176 days of fishing with a catch of around 2 kg / day. The total number of catches of mangrove crabs in one year reaches 176 kg / year, so the net income value of mangrove crab catchers in the Garongkong hamlet in the mangrove forest area is Rp.4,400,000 / year.

As for calculating the direct benefits of crabs in Lawallu Village, in one year, fishermen catch mangrove crabs for 471 days. The catch per day can reach 3 kg / day. The total catch of mangroves in a year reaches 484 kg / year. Net income from the catching of crabs in Lawallu Village is IDR 16,120,000 / year.

Value of Benefits of Collecting Firewood

Utilization of mangrove branches and twigs for firewood as a source of energy is usually done by

people who live or live around mangrove forests. At present the value of using mangrove wood, which is usually used as firewood, is no longer used by the people of Garongkong and Lawallu villages. This happens because of the awareness of the community or the community to obey the rules set by the Barru District government that the community is prohibited, cut or take wood from mangrove trees. In this research, the value of mangrove wood utilization is still calculated so that the community can compare the value of the use of wood used as firewood by stabilizing the value of using mangrove wood into firewood from other areas close to this research area.

In the Tahang study (2018), the community in Sinjai District still had a community that used mangrove wood as firewood. The economic value of mangroves for firewood is derived from the amount of firewood used by household rumors that are used instead of oil stoves or gas stoves. In the study, for areas that, as mangrove wood as firewood, were still in the area of East Sinjai, it was assumed that the number of households using firewood was 10% of the total respondents (KK) of 350 households. Umlah it could have been smaller or larger than what happened in the field. According to the results of surveys and interviews conducted by researchers, the amount obtained in collecting firewood for consumption as a substitute fuel is IDR 95,000. thus the value of mangroves for firewood in the eastern Sinjai mangrove forest area is Rp.400,400,000 per year.

Indirect Benefits of Mangrove Resources

Indirect benefits are values that indirectly feel benefits, can be in the form of things that support direct use value. Indirect benefits of mangrove forests in Barru District are physical and biological benefits. Indirect physical benefits are as a barrier to coastal abrasion which is estimated through replacement cost by making beach concrete for break water. The results obtained are based on the replacement cost of the breakwater value, referring to the estimation made by Aprilwati (2001), namely that the cost of constructing a breakwater 'size 1 mx 11 mx 2.5 m (length x width x height) with 10 year endurance of Rp4,153,880.00.

For the Garongkong region, making waves holds using materials such as cement, rock, sand and concrete steel. Costs incurred for making waves or break water in the Port of Garongkong, Garongkong Village amounting to Rp17,570,176.18. The average break water height in Garongkong Village is around 2.5 meters.

Value of indirect benefits = 1,581 m x Rp17,570,176.18

= Rp27,778,448,548.- Or

= Rp. 2,777,844,855 / year

The indirect benefits of mangroves as a barrier to the waves

Benefits of Options

The benefits of choice are approached by the value of biodiversity (biodiversity). According to Suryono (2006), the choice of benefits is a type of utilization that reflects the value of biodiversity (biodiversity) that can be captured from the presence of mangrove forests. The choice value is estimated based on the biodiversity value provided by the mangrove forest ecosystem according to the results of Ruitenbeek (1992) in Rivalda (2017), which is US $ 1,500 / Km2 / Year or US $ 15 / Ha / Year or Rp223,770 / Ha / year (US $ 1 = Rp. 14,918 at the time of the study). The value of selected mangrove forest ecosystems in Garongkong and Lawallu Villages can be explained in the table:

Table 3. Benefits of Mangrove Options in Garongkong Hamlet and Lawallu Village

Research Location	Biodiversity value(Rp/Ha/Year)	The large of Magroves forests	Value of Option
Garongkong	223,770	30	6,713,100
Lawallu	223,770	65	14,545,050
Total	**223,770**	**95**	**21,258,150**
Average	**223,770**	**47.5**	**10,629,075**

Source : Primary Data, 2018

The table above shows that Garonkong Hamlet has a chosen value of IDR 6,713,000 per year. While the value of choice for the village of Lawallu is Rp14,545,050. The difference in the value of choice between the two locations is due to the difference in the size of the mangrove forest area, where Lawallu village has a larger area of mangrove forest compared to Garongkong Hamlet. which is 65 Ha. In contrast, the mangrove forest in Garongkong Hamlet is the smallest area with an area of only 30 hectares of mangrove forest.

Benefits of Existence

The benefits of the existence of mangrove forests in Garongkong Hamlet and Lawallu Village, are obtained by valuation techniques based on surveys to find out the

willingness to pay or community WTP (Willingnes to Pay). Based on the results of interviews with the responbden, it is known that those who use mangrove forests directly are those who work as fishermen with the ability to pay around Rp. 40,000 to Rp. 50,000 per year. The results of interviews and surveys in Garongkong and Lawallu Villages are tabulated to get the total value of PAPs, average and value of existence benefits per individual per year and value of existence benefits per year

Table 4. Benefits of the existence of Mangroves in Garongkong and Lawallu Villages

Respondent Indicator	**Garongkong Hamlet**	**Lawallu Village**
Respondent	38	17
Large of Mangrove firewoods	30 ha	65 ha
Total Value WTP	**Rp1.700.000**	**Rp760.000**
Average WTP	**Rp44.736**	**Rp44.705**

Souce: Primary Data, 2018

The table above shows that respondents from Garongkong sub-village have the highest value of being Rp.1,700,000 per year with an average PAP value of Rp.44,736. Whereas for Lawallu Village, it has a low existence value of IDR760,000 per year with an average WTP value of IDR44,705. the difference between the number of PAPs in the two locations is based on the highest number of families or households directly utilizing the mangrove forest as a place to make a living. Garongkong Hamlet has 38 families who directly utilize mangrove forests, while in Lawallu Village there are 17 households who use mangrove forests directly.

Total Economic Value

The results of the identification and calculation of all benefits are the total economic value obtained from the mangrove forests in the Garongkong and Lawallu villages. Identification of the benefits obtained in this study include direct benefits, indirect benefits, benefit benefits, and the benefits of existence. The direct data found in both locations are the direct benefits of fisheries (fish, crabs, and squid). Indirect benefits calculated are ecological benefits (as a breakwater).

Table 5. Total Economic Value of Garongkong Hamlet and Lawallu Village

type	**Total Value of Benefits**		**Average Benefits**	
	Garongkong	**Lawallu**	**Garongkong**	**Lawallu**
Direct Benefits	1,328,623,842.00	844,521,627.00	60,985,961.95	35,354,240.00
Indirect Benefits	2,777,844,855.00	4,153,880.00	2,777,844,855.00	4,153,880.00
Option Benefits	6,713,100.00	14,545,050.00	223,770.00	223,720.00
Existence Benefits	1,700,000.00	760,000.00	44,736.00	44,750.00
Total	**4,114,881,797.00**	**863,980,557.00**	**2,839,099,322.95**	**39,776,590.00**

Source : Primary Data, 2018.

Based on the table above shows the total economic value in the two regions, namely Garongkong Hamlet and Lawallu Village. In Garongkong Hamlet, the total economic value obtained is IDR 4,144,881,797 with an average benefit of IDR 2,839,099,322.95. Whereas for Lawallu Hamlet, the total economic value obtained is IDR863,980,557 with an average total economic benefit of IDR39,776,590.

Multiplier Effect

Garongkong seaport / bulk port is the construction of Makassar's main port which is projected as a non-food dry bulk port such as cement, coal, clinker, etc., and the liquid bulk terminal. Before the loading / unloading activity of non-food dry bulk was in Soekarno Harbor, Makassar. Non-food dry bulk unloading activities will be moved from those originally located at Soekarno Port to Garongkong with the aim that the activity of loading and unloading existing dry bulk food (wheat flour, etc.) is separated from the activity of loading and unloading non-dry bulk food (cement, etc.) so as to produce healthier products and environments.

The port activities have an economic impact on the people who live or live near the port. The impact obtained by the community is the improvement of the road to the port. The road is the first road that provides access to the port, through the local residents before

making a road that connects directly to the trans Sulawesi road. In addition to road improvements, the port has another impact on society, namely the opening of employment opportunities. Open employment opportunities are as a docker and business opportunities are open such as grocery stores that are open around the port area.

Direct Impact

The direct impact is the total Non-Tax State Revenue (PNBP) obtained from the Garongkong port. Non-Tax State Revenues (PNBP) consist of several types of deposits, namely Sign / Navigation Services, Shipping Services, Ports Services, and the last one is Sea Transportation Services. In the services of signs / navigation total revenue in 2017 that is Rp257,366,748. For shipping services, the total revenue generated by the Garongkong port is Rp39,329,811. As for port services, total revenues in the year amounted to Rp2,368,627,666. As for sea transportation services, the total revenue generated for the Garongkong port is Rp54,016,364. The total of each deposit included in the Non-Tax State Revenue (PNBP) in 2017 is IDR 2,219,340,589.

Indirect Impact

The indirect impact is the wage of labor obtained from the loading and unloading activities of ships leaning at the Port of Garongkong. Workers at the Port of Garongkong numbered 105 people and as many as 53 respondents were respondents. Workers are divided into 6 groups and each group consists of 17-18 people. Work is divided into three shifts in one day, i.e. the first shift is at 08.00 - 16.00, for the second shift at 16.00 - 00.00, and the third shift at 00.00 - 08.00. The labor employment system at the Garongkong Port is using a wholesale system. Workers' wages or salaries will be given equally to each group and the group leader will share equally with the workers who were present at that time. The number of opinions or labor costs is IDR 38,700,000 with an average monthly wage of IDR 730,188.

Subsequent Impact

Follow-up impacts are exchanges issued by workers as cash flows obtained from direct and indirect impacts. The expenditure consists of labor expenses while working at the port location. Costs incurred for consumption costs amounted to Rp1,486,000 per month with an average expenditure of Rp.43,705 per month. Expenditures for consumption are only in the form of cigarettes and only 34 people consume cigarettes while working. For the consumption of lunch and dinner while working at the Port, workers choose to eat at their respective homes for their expenses. For coffee and mineral water while working, all are borne or have been provided by the company.

Multiplier Effect Results

The Multiplier Effect is used to calculate the economic impact caused by reclamation in Garongkong. Calculation of the Multiplier Effect can be seen in the appendix:

Table 6. Multipler Effect

Kriteria	Nilai	Keterangan
Keynesian Income Multiplier	3.07	Economic impacts that occur provide a large economic impact on reclamation activities because the value of the Keynesian Income Multiplier obtained is greater than 1 (≥1).
Ration Income Multiplier I	1.01	The economic impact is said to have a large impact because the value of Ration Income Multiplier I and Ratio Income Multiplier II is greater or equal to one (≥1).
Ratio Income Multiplier II	1.01	

Source: Primary Data, 2018

Based on the calculation of the results of the multiplier, the economy of reclamation provides a real and large economic impact seen from Keynesian Income Multiplier, Ratio Income Multiplier I, and Ratio Income Multiplier II which are quite high. The Keynesian Income Multiplier result of 3.07 means an increase in spending every Rp10,000, will increase Garongkong Port revenue and labor costs by Rp30,700. While the results of the Ratio Income Multiplier I of 1.01, which means increasing the income of the Garongkong Port by Rp10,000, will increase labor income by Rp10,100. The results of the Ratio Income Multiplier II of 1.01, which means an increase in income of the Garongkong Port by Rp10,100, will have an impact on direct impacts, indirect impacts, and subsequent impacts (Garongkong Port revenue, labor wages, and consumption expenses) by Rp10,100.

IV. CONCLUSION AND RECOMMENDATION

Conclusion

The conclusions from the results of research on the analysis of economic benefits in the activity of

reclamation and conversion of coastal land in Barru District are as follows:

1) The total economic value in the Garongkong hamlet is Rp4,114,881,797 with an average value of Rp2,839,099,322, while for Lawallu village it has a total economic value of Rp863,980,557 with an average value of Rp39,776,590.
2) The economy from reclamation has a real and large economic impact seen from Keynesian Income Multiplier, Ration Income Multiplier I, and Ratio Income Multiplier II which are quite high. The result of Keynesian Income Multiplier is 3.07. The result of Ratio Income Multiplier I is 1.01 and the Result Ratio Income Multiplier II is 1.01.

From the results of the calculation of the total economic value, the value of economic benefits and the Multipler Effect, it can be concluded that the reclamation activity can have a positive impact on the economic improvement of the community around the reclamation area.

Recommendation

Considering that reclamation is more profitable than land conversion, the government should pay more attention in the form of socialization, providing counseling to communities around the reclamation area to be able to improve the economy by utilizing available resources. Provision of assistance in the form of capital to provide production facilities for business development can be done by the local government.

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Eriobotrya japonica (Loquat) juice production parameters and their effect on sensory attributes and phenolic content

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***Abstract*—.** *The aim of this study is to assess the juice production potential of a highly perishable fruit, namely loquat and the effect of basic thermal and chemical treatment on the sensory attributes and phenolic content. The scarcity of loquat-based products on the market limits its consumption to raw loquat fruit. It is highly perishable and thus quickly loses its postharvest commercial quality. Loquat transformation into juice would increase the pellet of loquat-based products. Loquat fruit to loquat juice conversion value ranged between 1.79, 1.78, 2.41 and 2.39Kg of loquat fruit to produce 1kg of diluted fresh loquat juice, diluted fresh loquat juice with citric acid, pasteurized diluted loquat juice and pasteurized diluted loquat juice with citric acid respectively. The dilution factor was 0.2. All the Sensory attributes; taste, texture, appearance, aroma and overall-acceptance were significantly higher for both un-pasteurized loquat juices when compared with the pasteurized ones. Total Soluble Solids (TSS) values and TSS/Titratable-Acidity of the two pasteurized loquat juices were significantly higher than those which were not, given that water-activity and pH did not differ. TSS was significantly and negatively correlated with all the sensory attributes. Finally yet importantly, the phenolic content of the untreated fresh loquat juice scored significantly the lowest while the phenolic effect of the other juices did not differ significantly from each other. When the phenolic content per100gr TSS where the loquat juice with 1% citric acid scored significantly the highest than all of the other juices which did not differ significantly from each other.*

***Keywords*— Loquat, Fruit to juice conversion value, Total Soluble Solids, Phenolic content, Water activity.**

I. INTRODUCTON

Loquat (*Eriobotrya japonica Lindl*) belonging to the family Rosaceae and subfamily Maloideae, is a subtropical evergreen fruit tree and very well adapted to temperate areas [1]. Loquat Fruits are spherical or oval in shape, possess orange or yellow color, with soft and juicy flesh and a tough thin skin. Loquat is well adapted, cultivated and commercially spread in many regions although in the Mediterranean countries; it is underutilized for its production and thus consumption [2] [3]. Loquat is non-climacteric fruit [4] [5] the fruits are not suitable for storage and transportation and have a relatively short postharvest life. In the. The fruit-ripening period in Mediterranean region is concentrated in a few months of the year: March, April, and May [6].

Loquat fruit is popular for its healthiness, juiciness taste, and high content of phytochemicals such as carotenoids and flavonoids [7]. Loquat fruit is consumed largely as fresh fruit, but recently significant amount of work is done to induce it as a major ingredient in various processed food products, such as jellies and jams [8].

The development of loquat fruit has two stages: the growth stage, and maturation stage with ripening-related changes [9] [8]. The quality of loquat fruit is closely related to sugar accumulation and Total Soluble Solids/Titratable Acidity (TSS/TA) ratio [10]. The extractable juice rate decreases after low-temperature storage, and finally In the

last phase of loquat fruit senesces there is loss of flavor and taste, and gradual decrease in juiciness until a dry and firm texture -leather like- is reached [9]. Ethylene and CO2 production gradually decline during fruit maturation [11] [12].

Parameters like total acids [13], total soluble solids (TSS) and their ratio (TSS/TA) are very important in determining fruit quality [14] [15]. The high quality loquat fruits possess total soluble solids more than 12 Brix. The major soluble sugars in loquat fruit are fructose, glucose and sucrose and the major sugar alcohol is sorbitol [16]. Good flavor of loquat is closely related to the ratio between sugar and acid. Titratable acidity [13] of loquat fruit with good taste ranges from 0.3 to 0.6%. Titratable acidity [13] of loquat fruit with good taste ranges from 0.3 to 0.6% [17]

Aroma compounds are also important in contributing to the unique flavor of loquat fruit. The most potent of the aromatic compunds is phenylacetaldehyde [13]. Color/appearance, is the visible marker of loquat fruit ripening level and mainly the color of peel and flesh would result in juice color. Lutein and β -carotene were the major carotenoids in the peel of both red- and white-fleshed cultivars and β -cryptoxanthin in some red-fleshed cultivars, and β -carotene were the most abundant carotenoids in the flesh [18]. In addition, phenolic compounds, including several hydroxycinnamic acid derivatives and fl avonoid glycosides, were identifi ed from loquat fruit, and these can serve as antioxidative agents of excellent value for human health [19].

Loquat juice might be a food ingredient that provides flavor and freshness. It might also reduce the waste due to degradation of the very high perishability of loquat fruits even in fridge.

II. MATERIALS AND METHODS

2.1. Loquat fruits

In our study, loquat fruits (*Eriobotrya japonica Lindl*) were collected from orchards southern of Lebanon at around 140 days post anthesis.

2.2. Physico-chemical properties

Juicer: Excel Pro Juicer Silver JE880 (Kenwood) was used.

Brix Value: Brix Value was measured using Portable hand held RFM700 refractometer (Bellingham and Stanley LTD. United Kingdom).

Weight determination: Weight was measured using Portable electronic balance Model 727 was used to measure the weight with an accuracy of ±1 gr (Jata Hogar).

pH: Microcomputer based pH /conductivity /TDS /salinity and temperature pocket meter Model pH/EC80 was used to measure the pH (Jenco VisionP).

Titratable Acidity: TA was determined in triplicate by titration with 0.1 N NaOH up to pH 8.1 and expressed as g/100g malic acid, using phenolphthalein as indicator [20].

Volume Determination: 10mL glass graduated cylinder, with sub gradations of 0.1mL (Graduated cylinder, tall form, BLAUBRAND®, class A, Boro 3.3, DE-M).

Water activity: It was determined using Pawkit water activity meter. Samples were flattened to cover the bottom of the cup and then water activity was measured at room temperature [21].

2.3. Procedure

After having the loquat fruit they were deseeded and juice was extracted resulting in Fresh loquat Juice (FLJ). The loquat juice produced was diluted following this ratio: 250g water per 1 kg juice. This was done to be similar to recommended Brix Value between 10 to 11 similar to Honeydew melon and that of orange juice [22] [22]. Then this juice resulted in four different parts. One diluted loquat juice (DLJ), two diluted loquat juice plus citric acid (1%) (DLJCA), three pasteurized loquat diluted juice (PDLJ) and last but not least part four being pasteurized diluted juice plus citric acid (PDLJCA) (**Fig**. 1).

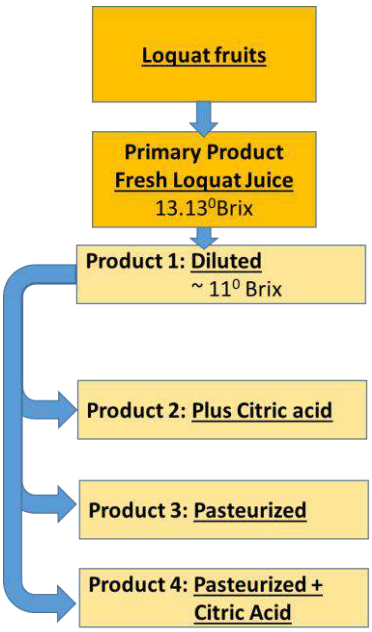

Fig.1 Summary of study flow

Each one kg of loquat fruit gave around 445gr fresh juice and 555gr leftovers. Please note that the pasteurization procedure was 85^{0}C for 7 minutes similar to that used by Hurtado [23].

2.3. Sensory Analysis

The sensory attributes attained from 60 taste panelists include: taste, with 1 having worst taste and 9 having the best; texture, with 1 having the worst texture and 9 having the best; appearance, with 1 having the worst and 9 having the best appearance; aroma, with 1 having no aroma and 9 having best aroma; and overall acceptability, with 1 having lowest acceptability and 9 having the highest acceptability. In addition to that, the sensory average score of each loquat juice was calculated by taking the mean of the different sensory attributes.

2.4. Phenolic content

Phenolic content was done using spectrophotometer method - HACH™ method # 8047 [24]. The 4-aminoantipyrine method measures all ortho- and meta-substituted phenols.

2.5. Statistical analysis

All tests and analysis were run in triplicates. General linear model performed via SPSS (statistical Package for the Social Sciences, version 17.0) was used to study the difference between the physicochemical properties and the score of the sensory attributes of the four different products. Least significant difference was used for mean separation of the physicochemical properties while Bonferroni test was used for the mean separation of the sensory attributes,

Furthermore, partial correlation was applied between the different sensory attribute and the physicochemical properties taking the type of product as a control variable.

TSS was significantly and negatively correlated with all the sensory attributes while other physicochemical properties were not significantly correlated with them.

III. RESULTS

3.1 Physicochemical

3.1.1 Water Activity (aw) and pH of the fresh loquat juice and that of the four products

The aw and the pH of the fresh juice and the four products did not differ significantly. All the pH-s were lower than 4.6 thus they are in the high acid product category and there was no significant difference between the pH of fresh juice and the different products. The aw of the different juices was around 0.96-0.97 (Table 1).

Table 1 Water activity and pH of Loquat Juices

	pH	Water Activity
	Mean ± SE	Mean ± SE
FLJ	3.60a ±0.13	0.97a ±0.01
DFLJ	4.00a ±0.16	0.96a ±0.01
DFLJCA	3.60a ±0.16	0.96a ±0.01
PDLJ	3.97a ±0.16	0.97a ±0.01
PDLJCA	3.57a ±0,16	0.97a ±0.01

- Within Columns, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L:loquat, J: juice; P: pasteurized, CA: Citric Acid;

3.1.2 Loquat juice conversion values

It was recorded that 2.22 loquat fruits were needed to produce 1kg of FLJ which was significantly lower than the conversion values of the pasteurized loquat juices with and without citric acid and significantly higher than diluted fresh loquat juices with and without citric acid (Table 2). This was mirrored in the kg juice per kg fresh loquat juice where the diluted loquat juice with and without citric acid were significantly lower than those of the pasteurized loquat juice with and without citric acid (Table 2).

Table 2 Different conversions to Loquat Juices

	Kg juice / kg loquat fruit	Kg juice / kg Fresh Juice
	Mean ± SE	Mean ± SE
FLJ	2.22a ±0.04	1*
DFLJ	1.79b ±0.05	0.8a ±0.01
DFLJCA	1.78b ±0.05	0.8a ±0.01
PDLJ	2.41c ±0.05	1.08b ±0.01
PDLJCA	2.37c ±0,05	1.06b ±0.01

- Within Columns, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: loquat, J: juice; P: pasteurized, CA: Citric Acid;
- *: It is the primary juice from which all other juices are done

3.1.1 Total Soluble Solids (TSS), Titratable acidity (TA) and TSS per TA

The total soluble solids was measured in terms of ^{0}Brix. The diluted fresh loquat juices with and without citric acid had the significantly lowest TSS followed by the TSS of the fresh loquat juice which in turn had a significantly lower TSS than that of the pasteurized loquat juices with and without the addition of citric acid them possessing the significantly highest TSS (Table 3).

The diluted fresh loquat juices with and without citric acid had the significantly lowest TA followed by the TA of the fresh loquat juice which in turn had a significantly lower TA than that of the pasteurized loquat juices with and without the addition of citric acid them possessing the significantly highest TSS (Table 3).

As for the TSS/TA values, those of the diluted fresh juice with and without the addition of citric acid were significantly the highest compared to fresh loquat juice, pasteurized loquat juices with and without citric acid addition, which did not differ significantly from each other (Table 3).

Table-3 Total Soluble Solids (TSS), Titratable Acidity (TA) and TSS/TA of the different loquat juices

	TSS (^{0}Brix)	TA (g/100gr)	TSS/TA
	Mean ± SE	Mean ± SE	Mean ± SE
FJ	13.13a	0.643a	19.371a
	±0.25	±0.014	±0.016
DFJ	10.67b	0.547b	19.519b
	±0.33	±0.016	±0.01
DFGCA	10.33b	0.529b	19.544b
	±0.33	±0.016	±0.018
PDJ	14.33c	0.742c	19.319a
	±0.33	±0.016	±0.018
PDJCA	13.67c	0,706c	19.347a
	±0.33	±0.016	±0.018

- Within Columns, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: loquat. J: juice; P: pasteurized, CA: Citric Acid;

3.2 Sensory attributes

3.2.1 Taste and aroma

The taste scores of the fresh loquat juice and the fresh diluted loquat juice with and without citric acid did not differ significantly from each other and scored significantly higher than the pasteurized loquat juice and the pasteurized loquat juice with citric acid (Fig. 2). Note that the fresh loquat juice and the diluted fresh loquat juice with and without citric acid addition had a score ranging from 7.26 till 8.00 while the pasteurized loquat juices with and without citric acid addition scored below 5 (Fig. 2).

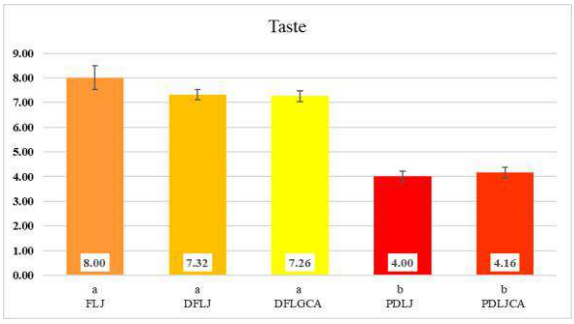

Fig.2 Taste scores of the different Loquat juices

- Within category means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: Loquat, J: juice; P: pasteurized, CA: Citric Acid;

As for the aroma scores the fresh loquat juice scored significantly the highest followed by the diluted fresh juice with and without citric acid addition, which scored significantly higher than pasteurized loquat juice with and without citric acid addition (Fig.3). Furthermore, the fresh loquat juice and the diluted fresh loquat juice with and without citric acid addition had a score ranging from 6.04 till 8.26 while the pasteurized loquat juices with and without citric acid addition scored below 5 (Fig. 3).

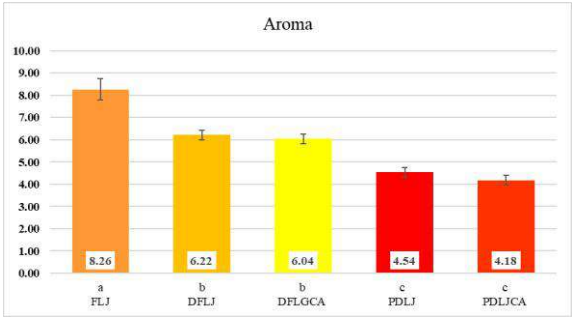

Fig.3 Aroma scores of the different Loquat juices

- Within category, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: Loquat, J: juice; P: pasteurized, CA: Citric Acid;

3.2.2 Texture and appearance

The texture score of the pasteurized loquat juice with and without citric acid addition scores were significantly the lowest followed by those of the diluted fresh loquat juice with citric acid addition, then by the diluted fresh loquat juice and ending, in a significantly ascending order, with the fresh loquat juice (Fig. 4). In addition to that, pasteurized loquat juices with and without the addition of citric acid scored below 5, and those scores of fresh loquat juice, diluted fresh loquat juice with and without citric acid ranged from 6.08 to 8.37 (Fig. 4).

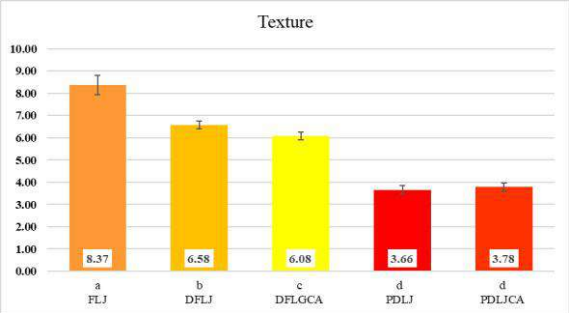

Fig.4 Texture scores of the different Loquat juices

- Within category, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: Loquat, J: juice; P: pasteurized, CA: Citric Acid;

As for the appearance sensory attribute, it was significantly the highest value for the fresh loquat juice, followed by the diluted loquat juice with and without citric acid addition, which possess significantly indifferent scores, ending with the significantly lowest values for the pasteurized loquat juice with and without citric acid addition (Fig. 5). In a similar pattern to the previous sensory attributes, the scores of the fresh loquat juice and diluted fresh loquat juice with and without citric acid addition ranged between 6.64 and 8.36 while those of pasteurized loquat juices with and without citric acid were below 5 (Fig. 5).

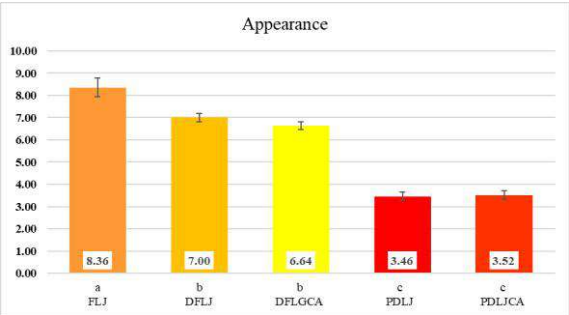

Fig.5 Appearance scores of the different Loquat juices

- Within category, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: Loquat, J: juice; P: pasteurized, CA: Citric Acid;

3.2.3 Overall acceptance and average score

The overall acceptance, which summarize the satisfaction of the panelist, followed the same pattern of the taste score values. The overall acceptance scores of the fresh loquat juice and the fresh diluted loquat juice with and without citric acid addition did not differ from each other and the values range from 7.25 to 8.33. Furthermore, the pasteurized loquat juice with and without citric acid addition overall acceptance scores did not differ significantly from each other, both scoring significantly lower than the fresh and fresh diluted loquat juices with and without citric acid with values ranging from 4.63 to 5.02 (Fig. 6).

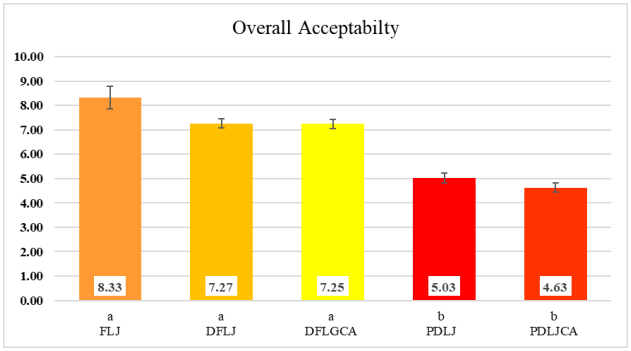

Fig.6 Overall acceptance of the different Loquat juices

- Within category, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: Loquat, J: juice; P: pasteurized, CA: Citric Acid;

The average score, which summarize the score of all the sensory attributes, of the fresh loquat juice did not differ significantly from the diluted fresh loquat juice, both average scores being significantly higher than the all the other loquat juices. The fresh loquat juice with citric acid average score was significantly higher than the pasteurized loquat juices, which had the significantly lowest average scores. The diluted fresh loquat juice and the diluted fresh loquat juice plus citric acids values, for overall acceptance and average score, were significantly higher than those values of the pasteurized diluted loquat juice and pasteurized diluted loquat juice (Fig. 6) (Fig. 7). The average score of the fresh loquat juice and the diluted fresh loquat with and without citric acid were between 6.65 and 8.39 and those of the pasteurized loquat juice with and without citric acid were below 5 (Fig.7).

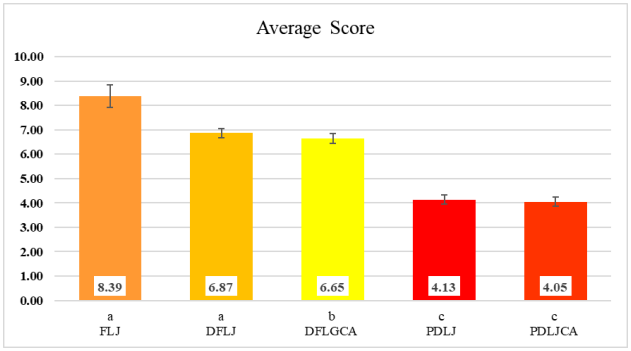

Fig.7 Average scores of the different Loquat juices

- Within category, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: Loquat, J: juice; P: pasteurized, CA: Citric Acid;

3.3 Phenolic Content

The phenolic content (mg/L) of the different loquat juices was significantly the lowest in the diluted fresh loquat juice followed by the fresh loquat juice, which was significantly lower than the rest of the loquat juices.

However, due to the different TSS of the juices, the phenolic content [25] was measured per 100gr TSS. This has showed that the phenolic content per 100 gr TSS was significantly the highest in the Diluted Fresh loquat juice with citric acid. There was no significant difference in this parameter in phenolic content per 100 gr TSS between all the other juices (Table 4).

Table.4 Phenolic content of Loquat Juice

	mg/L	Mg/100gr TSS
	Mean ± SE	Mean ± SE
FLJ	26.44a ±0.98	203.11a ±8.48
DFLJ	21.15b ±1.06	199.36a ±9.79
DFLJCA	28.91ac ±1.06	279.98b ±9.79
PDLJ	30.75c ±1.06	214.42a ±9.79
PDLJCA	29.90c ±1.06	219.05a ±9.79

- Within Columns, means with different alphabets are significantly different.
- D: Diluted, F: Fresh, L: loquat, J: juice; P: pasteurized, CA: Citric Acid;
- *: It is the primary juice from which all other juices are done

IV. DISCUSSION

The pH of the loquat juice in all forms, which did not differ significantly, ranged from 3.6 to 4.0 and thus it can be categorized as high acid fruit juice. This is in accordance to the pH recorded by Curi et al. who studied the processing potential of jellies from subtropical loquat cultivars [26]. The aw of the different Loquat juice also did not differ significantly and was in the range of 0.96 – 0.97.

The loquat fruit to loquat juice conversion values are an indicator of the amount of fruits needed per one kg of juice. As expected the diluted fresh loquat juices with and without citric acid needed the least amount of fruits followed by the fresh loquat juice and that the pasteurized juices. This reflected itself by the TSS of the different juices. Where the TSS of the fresh liquate juice is around 13.13^0Brix, which is an indicator of good quality fruits since it is higher than 12% TSS [17]. Furthermore, it is in accordance to Song et al. who studied the loquat fruit development and ripening process who reported similar Brix values at 140 post-an thesis days [10].

As for the TA the fresh loquat juice and the diluted fresh loquat juices with and without citric acid had a TA within the range of 0.53% and 0.64%. In addition, those of the pasteurized loquat juices with and without citric acid were around 0.7%. As for the fresh loquat juice, it is in accordance to the value reported by song et al. [10]. Furthermore, these values when combined with the sensory attributes results are in accordance with Tian et al. [17] who stated that TA of good tasting loquat fruit ranges from 0.3 to 0.6%. This might explain the sensory attributes results where the fresh loquat juice, diluted fresh loquat juice with and without citric acid scored significantly higher than the pasteurized loquat juices with and without citric acid. May be readjusting the TA of the pasteurized juices would improve their sensory scores.

When the TSS/TA is tested the interesting part that significance between the values of the fresh loquat juice and the pasteurized loquat juices with and without citric acid disappeared. This might be to the effect of the dilution, citric acid addition and pasteurization, which are factors that should be further investigated.

As for the phenolic content the diluted fresh juice had the lowest phenolic content, which can be explained by the lower TSS. However, by adding citric acid and with no pasteurization the phenolic content of the fresh loquat juice with citric acid was comparable to fresh juice and the pasteurized loquat juice with and without citric acid. Addition of citric acid might have induced chemical denaturation of oxidizing agent in the fresh juice hindering its deterioration. As for pasteurization it could have induced thermal denaturation of the oxidizing agent thus the effect of the citric acid was not noticed. However, looking at the fact that the addition of citric acid with no pasteurization resulted in the highest value of phenols per 100 gr TSS, with the other not significantly different, lead us to the assume that the pasteurized loquat juices with and without citric acid had the highest phenolic content is due to the significantly highest TSS. Pasteurization also resulted in significantly lower sensory scores on all level. Thus, pasteurization process of loquat juice should be further investigated to get the better temperature time combination with or without additives.

V. CONCLUSION

Loquat juice is a high acid juice. Pasteurizations process should be further investigated further and adjustment of the resulting juice to TA between 0.3 and 0.6 by water should be investigated. Addition of citric acid should be advised to ready to drink fresh juice to preserve the phenolic content

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Vaccination and deworming of foals: Owners' perspective

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Abstract— *Foals are susceptible to many infectious diseases and they should be treated and protected differently compared to adult horses. Objectives of this study were to investigate vaccination and deworming practices of foal owners in Finland. The questionnaire study was executed. Foal owners (n = 236) gave a response and 217 of them told that they vaccinate their foals against equine influenza and tetanus (combination vaccine) (88 %) and herpes (12 %), but not against rabies (1,8 %). About 8 % did not vaccinated their foal at all and a risk of being non-vaccinated was regionally distributed (p<0,05). Among foal owners deworming (99,2 %) preferred over vaccination (92 %). Foals were dewormed by taking regular fecal samples first (76%), but also routine treatments without samples were favored (22 %). Differences between foals of this study and horse population in general (horses of all ages) need to take seriously when conclusions are drawn. Different recommendations come from different veterinarians should be taken under further research.*

Keywords— ***equine influenza; deworming; foal; survey; vaccination.***

I. INTRODUCTION

Foals are especially susceptible to many infectious diseases. During their first weeks or months of life they encounter infectious agents such as *Rhodococcus equi*, rota virus and *E. coli* which are rare or less threatening among adult mature horses. Therefore, protection of the foal against specific infectious diseases that it is likely to encounter during the first few months of life, as its own immune system matures, relies heavily on postnatal absorption of specific antibodies and perhaps other factors that the dam has concentrated in colostrum during late gestation (Wilson et al., 2014). In general, vaccination of foals under six months of age is not recommended because maternal antibodies have been shown to exert a profound inhibitory effect on the immune response of foals to antigens, including those contained in vaccines. Foals less than six months of age consistently failed to mount serologic responses to inactivated influenza vaccines (van Maanen et al., 1992; Cullinane et al., 2001; Wilson et al., 2010) and cattle vaccines (Ryan & Giguére, 2010). In contrast, young horses can show increased cytotoxic cellular immune responses by modified-live herpes vaccines despite the presence of maternal antibodies (Ellis et al., 1997), and especially when 3-dose series with a multivalent vaccine are administered (Davis et al., 2015)

Most foals develop permanent immunity against *Strongyloides* not until by five or six months of age (Nielsen et al., 2014). On the other hand, it was found among thoroughbred foals that Strongyle counts increase and ascarid counts peak at 4,5 -5 months of age (Bellaw et al., 2016). Thus, in addition to external infectious diseases foals are threatened by internal parasites. Foal owners should comply deworming instructions recommended by their own veterinarian. As a result from one study among foals, the use of combination therapy of ivermectin-pyrantel against small strongyles and *parascaris* has been recommended (Luksovsky et al., 2013). Various drugs and drug combinations have been used among foals in Finland (Näreaho et al., 2011) and because of observed increase in anthelmintic resistance among horse population worldwide, it is recommended by Finnish veterinary practitioners that the efficacy of anthelmintic should be tested by taking fecal samples before and after the grazing season (Recommendation of Finnish Veterinary Practitioners, 2019; Horse Information Centre of Finland, 2019). Among foals, test results influence on only the choice of the anthelmintic and foals need to deworm in any case, regardless of the test results.

A consensus on current best deworming practice should achieve because there are many insufficient measures to reduce the development and spreading of anthelmintic

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resistance (Rendle et al., 2019). In Denmark, certain groups of horses such as foals, horses less than three years of age, pregnant mares and horses with clinical signs of parasitic diseases are treated with anthelmintics by 95 % of veterinarians without prior fecal analysis (Nielsen et al., 2006). Also horse owners' practices should be improved. Study from Italy shows that 85 % of horse owners do not ask for prior fecal examination and horses are routinely (94 %) and massively (61 %) dewormed (Papini et al., 2015). Similarly, Swedish horse owners told that only 1 % of them perform fecal egg counts on a regular basis (Lind et al., 2007). In Thoroughbred trainers in England not always based their choice of anthelmintic on veterinary advice (Earle et al., 2002) and in England, Scotland, Wales and Ireland many owners do not follow parasite recommendations available (Lloyd et al., 2000; Stratford et al., 2014; Elghryani et al., 2019). In contrast in Germany, both regular deworming management and selective anthelmintic therapy with less deworming times per year have been introduced (Simoneit et al., 2018).

In Finland it is estimated in one study that 85-95 % of horses are vaccinated at least against equine influenza (Koskinen, 2014a). However, foals below the recommended vaccination age in Finland (from five to seven months) were well represented in this survey and nothing is known about the vaccination of all foals (under 12 months of age). Risk factors for parasitic infections such as pasture hygiene and hygiene routines of housing (Aromaa et al., 2018), and farm size and frequency of horse movements (Hautala et al., 2019) have been revealed in Finnish conditions, but the internationally comparable study of deworming practices is neglected. The objective of this study is to investigate vaccination and deworming practices of foal owners in Finland. The goals are to complete previous vaccination study (Koskinen, 2014a) by the second questionnaire and to add worm control information for Finnish authorities.

II. MATERIALS AND METHODS

Questionnaire

Questionnaire of this study was based on previous survey among Finnish horse owners (Koskinen, 2014a) with some modifications. Questions were the responsibility of the author and a layout of current questionnaire was designed with collaboration of research center Kantar TNS (former Gallup of Finland). As background data geographical location of a foal home stable (Southern, Northern, Eastern, Western part of Finland or a foreign country), breed of a foal (thoroughbred, half-breed, warm-blooded/trotter horse, Finnish horse, pony or other), a number of foals per an owner and a number of horses in foal's home stable (under of over 10) were included. A basic questionnaire was based on both vaccination and deworming questions (Table 1) with recent recommendations given by Finnish Food Authority (Finnish Food Authority, 2019), Finnish Veterinary Practitioners (2019) and Horse Information Centre of Finland (2019). In the introduction section of the questionnaire owners were invited to take foal's passport close to their eyes before starting the survey because all of vaccination entries can be found in this passport.

Table 1 Survey questions

At what age I have vaccinated/will vaccinate my foal (several alternatives possible)
How many times I will vaccinate/have vaccinated my foal (several alternatives possible)
I will vaccinate/have vaccinated my foal against a) equine influenza (Equilis prequenza, Equip F, Duvaxyn IE Plus, ProteqFlu), b) against tetanus (Equilis, Tetanus), c) both against equine influenza and tetanus (Equip FT, ProteqFlu TE, Duvaxyn IE-T Plus, Equilis prequenza TE), d) against herpes rhinopneumonitis (Duvaxyn EHV 1,4, Equilis resequin, Equip EHV 1,4), e) against rabies (Rabdomun, Rabisin) (several alternatives possible)
I will vaccinate/have vaccinated my foal according to this recommendation
I will vaccinate/have vaccinated my foal according to the recommendations of the Finnish Trotting and Breeding Association (trotters in competitions)
I will vaccinate/have vaccinated my foal according to the recommendations of the Equestrian Federation of Finland (riding horses in competitions)
I will vaccinate/have vaccinated my foal according to the other recommendation
I do not vaccinate my foal
At what age I have dewormed/will deworm my foal (several alternatives possible)
How many times I have dewormed/will deworm my foal (several alternatives possible)
I will deworm/have dewormed my foal according to these recommendations, or one of them
I will deworm/have dewormed my foal according to the other recommendation (e.g. by using the old way in which all horses in stable will have been treated routinely)
I do not deworm my foal according this recommendation/these recommendations

Questionnaire distribution

In Finland, all foals must be registered in the Finnish Trotting and Breeding Association (Suomen Hippos) as soon as possible and within six months after birth. Firstly, announcement of birth must be submitted and secondly, identification process must be promoted. Thus, Suomen Hippos has a register of personal data such as contact details of foal owners, and before a distribution of the questionnaire, an agreement of transfer and utilization of personal data was to be obtained between Suomen Hippos and Kantar TNS.

This study was focused on foals, which meant horses under 12 months of age. In 2018, 2942 foals were born in Finland (The Finnish Trotting and Breeding Association (2019). For this survey, all foals born in 2019 were included. After the contract between Suomen Hippos and Kantar TNS had been concluded it was a duty of Kantar TNS to send phone messages or e-mails to foal owners and two reminder messages to those with no response. In total, invitations were sent to 1799 phone numbers. The survey was conducted in December 2019, at a time when vaccination of first foals of spring 2019 has begun (among six or seven months of age). The survey was coded and launched in early December before Christmas and respondents were given two weeks to reply.

Data analysis

Based on preliminary variable frequency tables with only a few observations in some background categories, such as a location (Northern part of Finland and a foreign country) and a breed (thoroughbred), these categories were modified. New categories of geographical location (Southern, Eastern and Western part of Finland or other location) and a breed (half-breed, warm-blooded/trotter horse, Finnish horse, pony or other) were established. For further statistical data analysis, SPSS statistics for Windows, version 25.0 (IBM Corp, Armonk, NY) was used. Vaccination and parasite control questions, classified into their own categories, were kept separated in logistic regression analysis. In this analysis, only first foal from each owner (if there were several foals) was included and odds ratios (OR) with lower and upper 95 % confidence intervals (CI) were calculated. This removal of second and following foals was due to an attempt to ensure that the foals had different owners and therefore, were independent of each other. The probability of being no vaccinated between categories of the explanatory variable was analyzed and as a result, one-variable regression model was achieved.

III. RESULTS

Overall, 236 responses were received. The number of foals among these owners varied between one to 16, being on average of 1,6 foals per owner (74 % had only one foal). All breeds were found albeit Finnish horses (35 %), warm-blooded/trotter horses (29 %) and half-breeds (20 %) were best represented. The foals were mainly located in Western Finland (39 %) in small (under 10 horses) stables (66 %) and they were almost all vaccinated and dewormed. Only two of these foals (0,8 %) were left without anthelmintic treatment and 19 (8 %) were not vaccinated.

Because only two foals left without anthelmintic treatment, the probability of being no dewormed between categories of the explanatory variable could not be calculated. Instead, related to vaccinations it was found that in Eastern Finland there was fivefold increased risk of being without vaccines compared to reference category Southern Finland (OR = 5,37, $p<0,05$), 95 % CI (1, 07, 27,08). All half-breed foals were vaccinated (100 %). Although the vaccine coverage left under 100 percent among other breeds, there were no significant ($p<0,05$) differences between breeds ($p=0,052$ between warm-blooded/trotter horses and Finnish horses).

Foal owners were unanimous in the importance of certain vaccines. Equine influenza vaccine with tetanus vaccine (combination vaccine) was given to foals with high level of coverage (192/217, 88 %). Surprisingly, also herpes virus vaccine was favored (27/217, 12 %). On the other hand, rabies vaccine was almost totally ignored (4/217, 1,8 %) and 14 (6,5 %) of the owners did not know or could not say their opinion. The majority of foals were vaccinated at six months of age (125/217, 58 %) and at a time of the survey the foals had received 1-3 vaccinations (92 + 78 + 41/217, 97 %). The majority of the owners complied with the recommendation of Finnish Food Authority (131/217, 60 %) when they vaccinate their foals. In addition, the recommendations of the Equestrian Federation of Finland and recommendations of the Finnish Trotting and Breeding Association were followed (49/217, 23 % and 33/217, 15 % respectively).

The majority of foal owners complied with deworming recommendations in which fecal sampling was included (177/234, 76 %). The old way with routine treatments or other recommendation were also supported (52/234, 22 %). At a time of the survey the foals had received at least one anthelmintic treatment (19/234, 8 %), but most commonly, three treatments (98/234, 42 %). Majority of the foal owners gave the first anthelmintic treatment at two months of age (166/234, 71 %), but also a practice with four or six months of age was observed (148/234, 63 % and 149/234, 64 %, respectively).

Below one (< 1) OR estimates were seen when values between deworming recommendations and an old routine anthelmintic treatment was calculated. They were not statistically significant (OR = 0,4, p=0,063 in Eastern Finland compared to reference category Southern Finland) and thus, an increased risk of using old routine treatment cannot be demonstrated. Respectively, with no significant analysis result (p=0,083), non-vaccination of a foal cannot be explained by non-deworming of a foal or vice versa, although a high OR estimate (12) between them was revealed.

IV. DISCUSSION AND CONCLUSIONS

The foals of this study are for the most part vaccinated and dewormed (92 % and 99,2 %, respectively). The majority of the owners complied with recommendations regarding current vaccination and deworming practices. However, these respondents are these foal and horse owners who see the importance of vaccination and fecal sampling before anthelmintic treatment. They also know how to answer (the correct answers from the researcher's perspective). On the other hand, they may not have experiences with vaccine side effects regarding their own equine; an important reason for non-vaccination of horses in previous studies (Koskinen, 2014a; Goyen et al., 2017) or they might have thought that despite the side effects vaccination is necessary for a good life of a foal. In Finland, it has been found that 85-95 % of horses of all ages are vaccinated depending on the number of horses per an owner (Koskinen, 2014a), so it can be estimated that the percentage shown here (92 %) is on the same line.

Survey results (92 %) were based on calculations in which only first foal from each owner (if there were several foals) was included. Estimation of 92 % is comparable to the highest vaccination level (95 % coverage score) among those horses, which owners have only one horse (Koskinen, 2014a) and the results that handling of more than three horses per week is a risk factor for non-vaccination (Goyen et al., 2017). Vaccination may become a cost issue when the number of horses increases. In addition to the owners' concerns about vaccine safety and effectiveness, in the list of reasons for non-vaccination costs are emphasized (Manyweathers et al., 2017).

Deworming responses among foal or horse owners has never before been collected in Finland and thus, it is difficult to say whether the high coverage of anthelmintic treatments in the current study can be generalized to the whole horse population. In addition, foals should be treated differently than mature horses. However, due to increasing resistance, also observed in Finland (Näreaho et al., 2011; Hautala et al., 2019), it is worrying that almost a quarter of the foal owners use anthelmintic against parasites without sampling and without a worry of increasing resistance. Based on the results of this study, the foal owners could be expected to be much more critical in using vaccines than in using anthelmintics. Perhaps the owners have not been sufficiently informed on reduced efficiency of anthelmintic medication and adverse effects of anthelmintic products.

Foal owners in Eastern Finland left more likely their foals unvaccinated compared to foal owners in Southern Finland. There are also long distances between horse stables in Eastern Finland (and lower horse density), compared to stables in Southern Finland and thus, fewer contacts between horses come from different venues. In one previous Finnish study (Koskinen, 2014b) differences between vaccinations (or vaccination markings) were found when geographical factors (Southern vs. Northern) were taken seriously so the difference between regions is not surprising. In this study, regional differences in use of anthelmintics (yes or no) could not be calculated, but in the next study, the prevalence of different worms in different regions should be compared to the use of anthelmintics and to the different instructions that veterinarians give to their customers. The prevalence of tapeworm infections in Finland, for example and the importance of tapeworms and deworming against tapeworms in horse population in general stimulates discussion from time to time. Some veterinarians believe that horses are not affected with tapeworms at all because the infection prevalence of tapeworms is low. It would be interesting to know, how opinions of veterinarians differ between different regions.

As a conclusion, by vaccination protocols of foal owners a herd immunity is induced. Secondly, a trend towards a more responsible anthelmintic use approach can be seen. The situations is not the same as in the early 2000s when many owners do not comply with medication recommendations (Lloyd et al., 2000; Earle et al., 2002) or a practice of veterinarians to give anthelmintics to large group of horses without prior fecal analysis (Nielsen et al., 2006). Most Finnish foal owners do not favor routine and massive anthelmintic control practice, still common in Italy in 2015 (Papini et al., 2015), but are closer to Scottish and German practice with fecal egg count analysis, reduced treatment frequency and selective anthelmintic therapy (Stratford et al., 2014; Simoneit et al., 2018). It may be due to increased knowledge of anthelmintic resistance or cultural change in the use of medicines in general.

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CONFLICT OF INTERESTS

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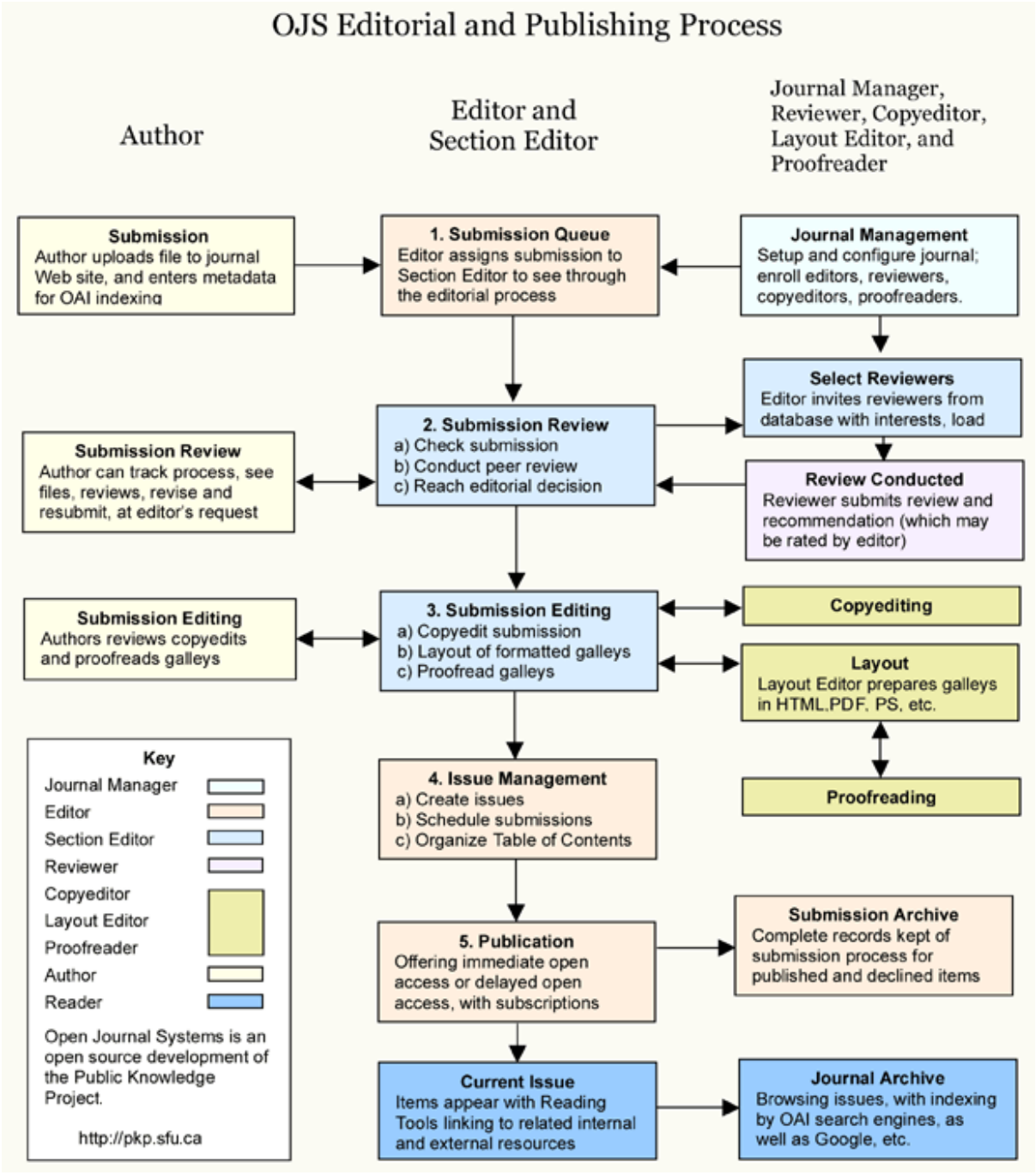

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