International Journal of Environment, Agriculture and Biotechnology
Vol-10, Issue-4; Jul-Aug, 2025
Peer-Reviewed International Journal
Journal Home Page Available: https://ijeab.com/
Journal DOI: 10.22161/ijeab

Impact of foliar chitosan and its oligomer on nitrogen metabolism in wheat seedlings *in vitro*

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Received: 09 Jun 2025; Received in revised form: 06 Jul 2025; Accepted: 10 Jul 2025; Available online: 17 Jul 2025

Abstract— *Chitosan and its oligomers are known to enhance plant defense and metabolism. In this study, a foliar application of 400 ppm chitosan was applied to 14-day-old wheat seedlings. Enzyme activity and physiological parameters were measured 8 hours post-treatment. Results showed significant increases in nitrate reductase (NR) and glutamine synthetase (GS) activities, along with elevated free amino acid (FAA) levels and chlorophyll content, compared to control. These findings suggest that chitosan enhances nitrogen assimilation and photosynthetic efficiency, demonstrating its potential as a plant metabolic enhancer.*

Keywords— ***Chitosan, Wheat, Foliar application.***

I. INTRODUCTION

Wheat (*Triticum aestivum* L.) is a cereal crop belonging to the family Poaceae, preferably grown in temperate climates and requires specific conditions for optimal growth, including cool temperatures during germination and warmer temperatures during grain filling (Porter *et al.,*1999). During the 2022-23 *Rabi*, India produced over 110 million metric tons (MMT) of wheat from 31.4 million hectares, accounting for approximately 37% of the nation's total food grain production with a record average productivity of 3520.7 kg/ha (FAO, 2023). Nitrogen (N) is a fundamental nutrient required for plant growth, particularly in cereal crops like wheat, which has a high nitrogen demand for optimal development and yield. As an essential component of amino acids, proteins, nucleic acids, and chlorophyll, nitrogen is involved in key physiological and biochemical processes including cell division, enzyme activation, and photosynthesis (Lea & Azevedo, 2006; Fageria & Baligar, 2005). In wheat, adequate nitrogen availability enhances vegetative growth, tillering, leaf area index, and promotes grain filling, ultimately leading to increased productivity and improved grain protein content (Good *et al.,* 2004). N acquisition efficiency of cereals is less than 50% of the N supply (Raun and Johnson, 1999), this value is lower than the N acquisition rate (60%) which is required for maximizing plant growth and yield (Sylvester-Bradley *et al.,* 2009). Currently, improving plant N absorption ability in soil may be a critical means to improve plant N efficiency and plant growth (Garnett *et al.,* 2013). Despite the widespread use of nitrogen fertilizers in wheat cultivation, nitrogen use efficiency (NUE) remains relatively low. It is estimated that only 30–50% of applied nitrogen is taken up by the plant, with the rest lost through volatilization, leaching, surface runoff, or denitrification (Raun & Johnson, 1999). Such losses not only reduce fertilizer efficiency and raise production costs but also lead to significant environmental issues, such as nitrate pollution of water bodies and emissions of greenhouse gases (Cowan *et al.,* 2019). Therefore, enhancing the efficiency of nitrogen use has become a critical goal in sustainable wheat production systems. In recent years, foliar application of nitrogen has been explored as an effective strategy to supplement traditional soil fertilization methods. Foliar feeding allows nutrients to be absorbed directly through leaf stomata and cuticular pathways, enabling rapid nutrient uptake and utilization, especially during peak demand periods or when root function is compromised (Fernández & Eichert, 2009). This method is particularly advantageous during late growth stages, such as grain filling, when root uptake becomes less

efficient and the plant requires an immediate nitrogen source to sustain photosynthesis and protein synthesis (Tahir *et al.,* 2024). Given the importance of nitrogen in wheat physiology and the potential advantages of foliar feeding, this study aims to evaluate the role of foliar-applied nitrogen sources in enhancing the growth, physiological traits, and yield performance of wheat plants. The findings may contribute to developing more efficient and sustainable nutrient management practices in wheat production. Since the Green Revolution, nitrogen fertilizers have significantly increased wheat yields; however, their overuse has caused environmental concerns such as soil acidification and nitrous oxide emissions (Tilman *et al.,* 2002; Harty *et al.,* 2016). This necessitates a shift toward sustainable and eco-friendly nutrient management practices. Chitosan, the second most abundant natural biopolymer, is a linear polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine units connected by β-(1–4) glycosidic linkages. It is derived through the deacetylation of chitin (Prajapati *et al.,* 2022). Chitosan contains two highly active functional groups, C6-OH and C2-NH_2, in its backbone, giving it a wide range of biological and physical properties. Additionally, chitosan has been highlighted for its exceptional elemental composition carbon (54.4–47.9%), nitrogen (7.6–5.8%), and oxygen (42.3–30.19%) making it a valuable organic source for plants (Prajapati *et al.,* 2022). The biological functionalities of chitosan can be further enhanced through hydrolysis, converting its native structure into chito-oligosaccharides (COS). COS offers superior solubility, bioavailability, and elicitor activity, outperforming bulk chitosan in promoting crop health, stress tolerance, and soil microbial interactions, making it a highly efficient candidate for next-generation sustainable agriculture (Ahmed *et al.,* 2015).

II. MATERIALS AND METHODOLOGY

2.1 Synthesis and characterization

Initially, 2% (w/v) chitosan was dissolved in 1% (v/v) acetic acid via electro spinning at 320 rpm until clear, homogenous solutions were obtained, and further chitosan-oligomers were prepared through a series of mechano-chemical processes (acid hydrolysis, oxidative degradation, fine-sieving /filtration, and centrifugation). The structural characteristics and stability parameters of the newly synthesized chitosan and COSwere studied and confirmed using dynamic light scattering (DLS). The Z-average size (hydrodynamic size) of the component particles, poly-dispersive index (PDI), and zeta potential were determined using a Zetasizer (ZS90, Malvern) at a constant temperature of 25 °C and a scattering angle of 90° during the DLS investigation.

2.2 *In vitro* seedling bioassay to measure real time impact on nitrogen metabolism

Wheat seeds were grown in a mini-glass house. Afterwards, seeds were sown in different seedling trays (10 seeds in each pot hole) filled with cocopeat and vermicompost mixture (1:1) in controlled light condition. Seedling trays (having 35 pot holes) were kept at mini-glass house set up. Regular watering was done to keep the germination area satisfactorily moisten. Foliar application of various forms of chitosan, oligomers were performed with 400 ppm concentration on 14-days old wheat seedling. Leaf samples were collected from mini glass house at 8h after foliar application to measure nitrate reductase (NR; EC 1.6.6.1), glutamine synthetase (GS; EC 6.3.1.2) using available protocols (Hageman and Hucklesby, 1971; Lillo, 1984). Likewise, leaf samples were also be used for the estimation of free amino acid and chlorophyll content (a and b) (Lichtenthaler, 1987) 8 h after foliar application.

2.2.1 Nitrate reductase activity

The activity of nitrate reductase (NR; EC 1.6.6.1) in leaves of 14-days old wheat seedlings grown in a mini-glass house was measured as described by Hageman and Hucklesby, 1971; along with in-house modifications. First, 1 g of fresh leaves were ground into powder using liquid N and 100 mM Tris HCl buffer (0.5 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$ and 1% PVP). After centrifugation at 10,000 rpm for 10 min, the supernatant was transferred into a reaction mixture containing a solution of nicotinamide adenine dinucleotide (1 mM NADH), Tris HCl buffer(25mM) and KNO3(100 mM). The mixture was incubated at 30°C for 30 min in the dark. After incubation, the NO_2^- produced pink colour, which was measured at 540 nm after addition of 1% Sulfanilamide in 3M HCl and 0.2% NED (N-1-naphthylethylenediamine dihydrochloride) were added to terminate the reaction.

2.2.2 Glutamine synthetase activity

Following Lillo, 1984; 1 g fresh samples taken from mini glass house grown plant leaves were ground into powder to determine the activities of glutamine synthetase (GS) (EC 6.3.1.2). After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was retained for reaction. The reaction mixture for GS was a solution containing 0.1M imidazole, 0.08M $MgSO_4$ $7H_2O$, 0.02M Glutamic acid and 58mM Hydroxylamine hydrochloride at pH 7. Then add 10mM ATP (pH of ATP solution should be just above adjust pH 7), after incubation at 30°C for 15 min, terminate the reaction by adding 1ml of $FeCl_3$ reagent (3.5% $FeCl_3.6H_2O$, 2% HCl, 2% trichloroacetic acid). The absorbance of the supernatant was measured at 540 nm for the calculation of GS activity.

2.2.3 Free amino acid content

Free amino acids were extracted from 14-days old wheat seedlings grown in a mini-glass house was measured according to (Rosen, 1957) 1g of fresh leaves were ground into powder using liquid N and 80% ethanol. After centrifugation at 10,000 rpm for 10 min, the supernatant was transferred into 0.8% ninhydrin reagent mix it well and kept in water bath at 100℃ for 20min, cool the sample at room temperature and the absorbance of the supernatant was measured at 570 nm and the enzyme activity was calculated using double-beam UV-VIS spectrometry.

2.2.4 Chlorophyll content

To extract chlorophyll from 100 mg of finely cut leaf fragments, a solution of 10 ml dimethyl sulfoxide (DMSO) was employed, and the mixture was subjected to a constant temperature of 65°C for a period of 3h. Occasional gentle agitation was performed one or two times during this interval to ensure complete chlorophyll extraction. Subsequently, the supernatant was meticulously collected, and its optical absorbance was measured at two specific wavelengths, namely 663 nm and 645 nm, employing a UV-VIS spectrophotometer. This analytical procedure adhered to the methodology detailed by Hiscox and Israelstam (1979).

Chlorophyll a, chlorophyll b, and total chlorophyll concentrations (expressed in mg/g) were then calculated employing the following precise formulas:

Chlorophyll a (Chl. a) = (12.7 × A663 - 2.63 × A645) × V / (1000 × W)

Chlorophyll b (Chl. b) = (22.9 × A645 - 4.48 × A663) × V / (1000 × W)

Total chlorophyll = (20.2 × A645 + 8.02 × A663) × V / (1000 × W)

Where,

V = represents the volume of the sample (10 ml)

W = corresponds to the weight of the leaf sample (100 mg)

2.3 Statistical analysis

The statistical analysis was used as post hoc test to determine the significance difference between the treatments at P=0.05 level. The analysis will be performed with JMP software version 12 (SAS, 2010) using Turkey Kramer HSD test.

2.4 Location of Research experiment

Mini-glass house experiment was conducted at the Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Udaipur.

III. RESULTS

3.1.1 Synthesis and characterization of chitosan and its oligomer

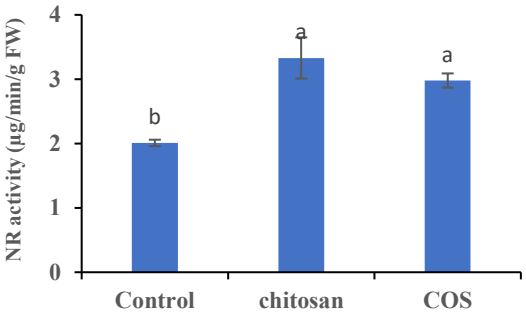
2% chitosan solution was formed by dissolving 2 g of raw chitosan flakes in to 100 ml of 1% acetic acid solution and further chitosan-oligomers were prepared through a series of mechano-chemical processes (acid hydrolysis, oxidative degradation, fine-sieving /filtration, and centrifugation). The DLS report and viscosity justified the synthesized complex structure as a stable formulation with satisfactory amount of component particles.

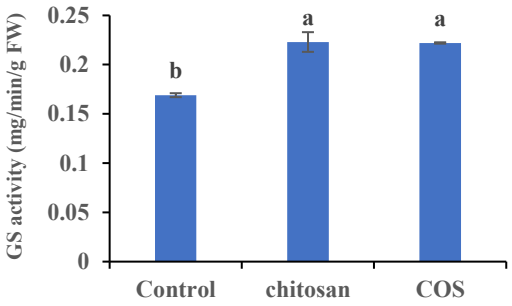
Table 3.1: Physico-chemical properties of chitosan and its oligomer

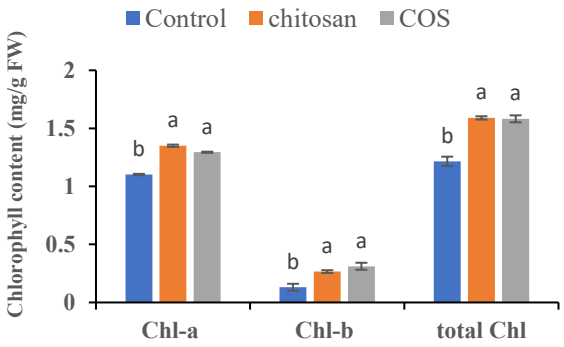
Chitosan forms	Zeta potential (mV)	Z Avg. (nm)	Polydispersity index (PDI)	Counts per rate (kcps)
Bulk chitosan	+94.1	26K	0.216	348
COS	+45.2	538.2	0.604	476.8

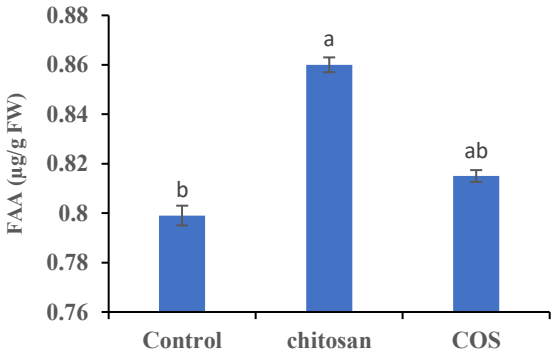
3.2 Bio-efficacy evaluation of chitosan and its oligomer on nitrogen metabolism in wheat

Foliar application of various forms of chitosan and COS was performed with 400 ppm concentration on 14-days old wheat seedling. Leaf samples were collected from mini glass house at 8h after foliar application to measure various enzyme activity. After 8 h NR activity increased in chitosan as compared to control (3.33 µg/min/g) treatment and control (2.01µg/min/g). After 8 h GS activity increased in chitosan as compared to control (0.233mg/min/g) and control (0.169mg/min/g). After 8 h FAA content increased in chitosan (0.86mg/g) and in control (0.799mg/g). After 8 h chlorophyll a increased in chitosan (1.35mg/g) as compared to control (1.102 mg/g) and chlorophyll b increased in COS (0.312mg/g) as compared to control (0.131mg/g) and total chlorophyll content increased in chitosan (1.59mg/g) as compared to control (1.22mg/g).









IV. DISCUSSION

The present study demonstrates that foliar application of chitosan and its oligomers at 400 ppm significantly enhanced physiological and biochemical parameters in wheat seedlings within 8 hours of treatment. Notably, NR activity showed a marked increase in chitosan-treated plants (3.33 µg/min/g) compared to control (2.01 µg/min/g), indicating improved nitrogen assimilation efficiency (Zang *et al.,* 2019). Similarly, GS activity also rose in treated samples (0.233 mg/min/g) versus control (0.169 mg/min/g), supporting enhanced ammonium assimilation into amino acids (Bernard *et al.,* 2009). Elevated FAA content in chitosan treatment (0.86 mg/g) further suggests improved nitrogen metabolism. Additionally, photosynthetic pigments, particularly chlorophyll a, b, and total chlorophyll, were significantly higher in treated plants, indicating enhanced photosynthetic capacity (Hadwiger, 2013). The increase in chlorophyll b in COS-treated plants implies potential oligomer-specific responses. These findings collectively highlight chitosan's role in enhancing nitrogen metabolism and photosynthesis under short-term application, likely via signalling-mediated enzyme activation and improved nutrient uptake (El Hadrami *et al.,* 2010).

V. CONCLUSION

The findings of this study clearly demonstrate that foliar application of chitosan and its oligomers at 400 ppm concentration significantly enhances nitrogen metabolism and photosynthetic efficiency in wheat seedlings under mini glass house conditions. The marked increases in NR and GS activities, free amino acid content, and chlorophyll levels within just 8 hours of treatment indicate a rapid physiological response. These enhancements suggest improved nitrogen assimilation and utilization, likely mediated by chitosan-induced activation of key metabolic pathways. Overall, chitosan and its oligomers show promising potential as eco-friendly plant growth regulator to improve crop nutrient efficiency and reduce reliance on synthetic nitrogen fertilizers, contributing to sustainable agricultural practices.

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