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RESEARCH ARTICLE

Adaptation in action: Plant defense dynamics in *Triticum aestivum* L. challenged by chemical elicitors

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Abstract

The present study was carried out with the aim of (i) evaluating the effect of chitosan on the free radical scavenging activity, ferric reducing antioxidant power (FRAP) activity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and chitinase activity in *T. aestivum* L. and (ii) evaluating the effect of different concentration of chitosan on enzyme activities such as phenylalanine ammonia-lyase (PAL) activity and beta- 1, 3-glucanase activity in *T. aestivum* L. To achieve these goals, the 15-days-old wheat seedlings were treated with different concentrations of chitosan, such as 1-4 mg/mL and further, the enzyme assays were carried out. The results obtained showed that treatment with 0.5 mg/mL of chitosan induced the maximum free radical scavenging activity and DPPH activity in the plant, while treatment with 1.0 mg/mL of chitosan induced the maximum FRAP activity. The response of wheat seedlings to an increase in oxidative stress was correlated with chitosan treatment. Additionally, treatment with 1.0 mg/mL of chitosan to the plant also showed maximum chitinase enzymatic activity in wheat seedlings. Chitosan treatment also elicited enzyme activities such as PAL and beta-1, 3-glucanases. Different concentrations of chitosan treatment showed increasing patterns of PAL activity from 0 hr, which peaked between 24 - 48 hr and thereafter, started decreasing post 72 hr. Similarly, chitosan treatments in wheat seedlings showed an increasing activity of beta-1, 3-glucanase from 0 hr till 48 hr and started decreasing at 72 hr. Overall, the findings showed that chitosan exhibits a protective nature against oxidative stress.

Keywords: chitinase; chitosan; elicitation; glucanase; PAL

Introduction

Wheat (*T. aestivum* L.) is a major staple crop, supplying approximately 20 % of global caloric and protein requirements for over 4.5 billion people (1). With an annual production of 600-700 million tons, it ranks among the most important agricultural commodities worldwide (2). Wheat is a valuable source of protein (8-20%), dietary fibre, B vitamins and essential minerals such as iron, magnesium and selenium (3, 4). Despite its global significance, wheat productivity is constrained by multiple environmental stresses, including salinity, water scarcity, heavy metal toxicity and both biotic and abiotic stresses, which can reduce yields by 20-40 % (5). Wheat contains many phytochemicals such as phenolic acids, phenolic acid oligomers, flavonoids, alkaloids, carotenoids, terpenoids, alkylresorcinols, tocopherols, tocotrienols, phytosterols, benzoxazinoids and many more compounds with phenolics, flavonoids and terpenoids being the major secondary metabolites found in the plant. They are produced by the phenylpropanoid pathway and are engaged in essential metabolic and physiological processes in plants, including stress tolerance (6).

Plant secondary metabolites are known to play an important role in stress induction and defense mechanisms (7). Some secondary metabolite systems are dynamic, responding to attack, infection or stress, while increased secondary metabolite accumulation is a part of an integrated defense strategy. Other components of the systems include the PR proteins, which have varied activities and these signalling responses further trigger the whole signalling cascade (8). During growth, plants experience various stresses that impact their metabolism, which can lead to variations in their yield, physiological state, nutritional and health benefits, which further leads to an increase in plant metabolites (9). Another condition under which secondary metabolites are accumulated is when the plants are subjected to various elicitors or signal molecules (10). Plant elicitors, also known as biostimulants or resistance inducers, prime plants by enhancing their metabolism, reinforcing their defenses and development, thereby combating stressed conditions (10).

The exposure to stress induces various physiological and metabolic changes in plants, which lead to alterations in the intracellular protein expression (11). Elicitors are natural or chemically synthesized substances that stimulate chemical defense in plants by producing phenolic compounds and activating a variety of defense-related enzymes. One such plant elicitor studied in wheat is salicylic acid (SA), which is known to enhance its enzymatic activity, growth, photosynthesis and water relations (12). Salicylic acid affects a plant's primary metabolism by regulating its nutrient uptake, water relations, photosynthesis, stomatal opening/closing and plant interactions with surrounding organisms (12). This plays a significant role in the development and resistance of plants. Various elicitors such as methyl

jasmonate, sodium salicylate, riboflavin (B_2), thiamine hydrochloride (B_1), 2,6-dichloropyridine-4-carboxylic acid and many more activate signalling molecules which directly activate the salicylic acid signalling pathway and further antimicrobial pathogenesis-related genes (13). Additionally, it also creates resistance against the fungus by increasing the activity of enzymes like superoxide dismutase (SOD) and peroxidase (POD) (14). Another example of a chemical elicitor is chitosan, which is a polymer formed from chitin, the second most prevalent biopolymer in nature after cellulose. Chitosan is a large cationic polysaccharide and is an abundant and economic organic compound obtained from seafood waste (15). Chitosan has remarkable biodegradability, biocompatibility and is non-toxic. Because of its cationic characteristics, chitosan easily adsorbs to plant surfaces, increasing the contact duration between the plant and the agrochemical. This makes it an effective plant growth accelerator and has been widely used for agricultural research (16). Chitosan is known for its antibacterial and antimicrobial properties and has good physicochemical properties. It also increases the rate of photosynthesis and nutrient uptake in plants (17).

Studies indicate that under stressed conditions, chitosan significantly improved growth indices such as germination rate, wet weight, root length, root activity and physiological indices such as SOD, POD and catalase (CAT), as well as the concentration of malondialdehyde (MDA) and chlorophyll (18). Enzymes such as chitinase, beta-1, 3-glucanase, POD and PAL (phenylalanine ammonia-lyase) are part of the overall array of enzymes that support plant defense by slowing pathogen growth (19). Chitosan is also known to reduce abiotic stress in plants by increasing the activity of antioxidant enzymes, including SOD, CAT and POD, leading to the regulation of reactive oxygen species (ROS) (20). These increased enzymatic activities have been known to reduce superoxide radicals, hydrogen peroxide (H_2O_2) levels in three different varieties of wheat cultivars and for reduction in membrane damage in the plant (21). Chitosan affects the defensive system of plants by accumulating ROS, expressing defense-related genes, activating pathogen-response proteins and producing phytoalexins, phytoenzymes and phytohormones (22).

The present study was undertaken to investigate the potential effect of the chemical elicitor chitosan on the antioxidant activities of wheat. Chitosan, as an elicitor, was also exploited to check its effect on various defense-related enzymes such as chitinase, PAL and beta-1,3-glucanase.

Materials and Methods

Procurement of germplasm

T. aestivum L. grains of the cultivar "496-Sabarmati" were obtained from Sahakar Biyaren Kendra, Karjan, Vadodara, Gujarat, India.

Establishment of wheat seedlings and study of germination

The wheat grains were cleaned to remove contaminants and then kept for germination on a piece of watered cotton in Petri dishes in the dark for fifteen days. To avoid drying, the seeds were watered every 12-18 hr. After 15 days, the germinated seeds were used for the assays. The germination percentage was calculated as follows:

$$\text{Germination Percentage (\%)} = \frac{\text{Number of seeds ger min a ted}}{\text{Total number of seeds}} \times 100$$

Chemical elicitation

Elicitation strategies

Shrimp shell chitosan was purchased from HiMedia and ground into a fine powder. Chitosan was dissolved in 0.25 N HCl and centrifuged for 15 min at 10000 rpm to eliminate any undissolved particles. The viscous solution was then neutralized with 2.5 N NaOH at pH 9.8 to precipitate chitosan. The precipitated chitosan was recovered by filtration. It was thoroughly rinsed with deionized water to remove salts and lyophilized, with the help of a lyophilizer (Thermo Star Laboratory, Lyophilizer). Chitosan stock solution (10 mg/mL) was autoclaved and then added to sterile, distilled water to achieve the necessary final chitosan concentrations of 0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2.0 mg/mL and 2.5 mg/mL (23). The 14-day-old seedlings were then sprayed with different concentrations of chitosan, prepared and then stored at -20 °C for future experiments. Distilled water served as control in the experiment.

Preparation of the enzyme extracts

The fifteen-day-old seedlings (one gram each) were weighed and crushed in a pre-chilled mortar and pestle with 3 mL phosphate buffer solution containing sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate (pH 7.4). The extract was centrifuged at 4 °C for 10 min at 3000 rpm and the resulting supernatant was utilized to perform antioxidant assays and enzyme assays (24).

Enzymatic assays

Free radical scavenging activity

The free radical scavenging activity was determined by adding 1.5 mM $FeSO_4$, 0.7 mL 6 mM hydrogen peroxide, 0.3 mL 20 mM sodium salicylate and varying concentrations of the extract and the reaction mixture was incubated for 1 hr at 37 °C (25). The absorbance was then measured at 562 nm. The capacity of the extract to scavenge free radicals was calculated using the following formula:

$$\text{Scavenging Percentage (\%)} = \frac{A_C - A_A}{Ac} \times 100$$

Where, A_C represents the absorbance of the control (the solvent without extract) at 0 min and A_A represents the absorbance of the sample after 2.5 min. Ascorbic acid, an analogue of vitamin C, was used as a standard for antioxidant activity.

Ferric reducing antioxidant power (FRAP) activity

The FRAP assay was done by adding 3.6 mL of the FRAP solution to distilled water (0.4 mL) and incubating at 37 °C for 5 min. Then, this reaction mixture was mixed with different concentrations of the plant extract and incubated at 37 °C for 10 min. The absorbance of the reaction mixture was measured at 593 nm (26).

For the construction of the calibration/standard curve, five different concentrations of ferrous sulphate ($FeSO4.7H_2O$), viz., 0.1 mM, 0.4 mM, 0.8 mM, 1 mM, 1.12 mM and 1.5 mM, were used and the absorbance values were measured as for the sample solution using a microcontroller-based UV-visible spectrophotometer (CL-1320 Chemiline).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH stable free radical scavenging activity was carried out by adding 3 mL of different concentrations of the plant extracts to 1 mL of a 0.1 mM DPPH solution in methanol. After 30 min of incubation at 37 °C, absorbance at 517 nm was measured against a control using a microcontroller-based UV-visible spectrophotometer (CL-1320 Chemiline) (27).

Ascorbic acid was used as the standard. The percentage of inhibition was estimated by comparing the absorbance values of the test samples with those of the control. The inhibition percentage (%) was obtained using radical scavenging activity (RSA) as follows:

$$\text{Scavenging Percentage (\%)} = \frac{A_C - A_A}{Ac} \times 100$$

Where, A_C represents the absorbance of the control and A_A represents the absorbance of the sample.

Chitinase assay

Five percent colloidal chitin (GRM1356, HiMedia, Mumbai, India) was prepared by dissolving in 0.05 M sodium acetate buffer (pH 5.0). The reaction mixture was prepared by adding 0.1 mL of chitin solution, 0.1 mL of 0.5 M sodium acetate buffer (pH 5.0), 0.7 mL of distilled water, 0.1 mL of the enzyme extract and was kept for incubation for 3 hr in the water bath at 37 °C. After the incubation, the reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent and the tubes were kept in a boiling water bath for 10 min. The mixture was then allowed to cool down for 5-7 min and then kept for centrifugation at 8000 rpm for 10 min. The absorbance was measured at 540 nm and compared to the control sample using a microcontroller-based UV-visible spectrophotometer (CL-1320 Chemiline). Chitinase activity was expressed as the amount of N-acetyl-D-glucosamine (NAD) released per hour per milligram of protein (mg NAD mL^{-1} h^{-1} mg^{-1} protein). The protein content of plant extracts was determined using Bradford's method (28).

Protein quantification by Bradford's method

The Bradford assay (29) relies on the binding of the dye Coomassie Brilliant Blue G-250 to the protein in the samples. A total of 300 µL of Bradford reagent was added to 10 µL of protein fraction (samples) and the reaction mixture was incubated for 5 min. After incubation, the absorbance was measured at 600 nm using an ELISA reader (Analytic Techniques Limited, Vadodara, Gujarat, India). Concentration of proteins was expressed as mg proteins per gram of dry weight (mg protein g^{-1} DW).

Phenylalanine ammonia-lyase (PAL) activity

The PAL activity was evaluated by the conversion of L-phenylalanine to trans-cinnamic acid. The reaction (30) mixture, to a final volume of 1 mL, consisted of 500 µmol of Tris-HCl buffer (pH 8.0), 100 µL of enzyme preparation and 6 µmol of L-phenylalanine for PAL assay (Sigma-Aldrich, Mumbai, India). The enzyme reaction was started by the addition of enzyme extract and after incubation for 60 min at 40 °C, the reactions were stopped by the addition of 50 µL of 5 N HCl. The amount of trans-cinnamic acid formed was determined by measuring absorbance at 290 nm using an ELISA reader (Analytic Techniques Limited, Vadodara, Gujarat, India). The enzyme activity was expressed in nmol (cinnamic acid) mg protein $^{-1}$ min^{-1}.

Beta-1, 3-glucanase activity

The activity of β-1,3- glucanase was performed with minor modifications to the original method (31). The samples were treated with 0.1 % (w/v) laminarin prepared in 0.05 M sodium acetate buffer (pH 5.2). The mixture was vortexed for 15 min at 37 ° C. The reaction was stopped by adding DNS reagent [consisting of 0.5 % (w/v) 3,5-dinitrosalicylic acid (Sigma) and 15 % (w/v) potassium sodium tartrate tetrahydrate (Sigma)]. The mixture was then kept in the water bath for 10 min. The absorbance was measured using an ELISA reader (Analytic Techniques Limited, Vadodara, Gujarat, India) at 540 nm against glucose as a standard.

Statistical analysis

The reported values represent the means of a minimum of three replicated measurements. Analysis was conducted using GraphPad Prism 8.4.2. A one-way analysis of variance (ANOVA) was conducted, followed by Tukey's Honestly Significant Difference (HSD) test to compare the means of different treatments. Data are presented as the mean of three replicates ± standard deviation, where $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ and ns represents a non-significant value.

Results

Establishment of wheat seedlings and study of germination

The germination of seed sets was monitored daily to check the percentage of seeds. The germination percentage of the seeds was found to be 95 % (Fig. 1(A-D)).

Chemical elicitation

Elicitation with chitosan

Fig. 2(A-D) illustrates the chitosan treatment effect on antioxidant activity and chitinase enzyme activity in wheat seedlings across different concentrations (0.5-2.5 mg/mL) compared to the untreated samples. Stress conditions usually increase antioxidant activities in plants. Similar results were found in this study as well, the DPPH free radical activity was seen highest at 0.5 mg/mL concentration at 62.22 % and as the concentration of chitosan increased, the DPPH antiradical activity decreased i.e. 53.46 % at 1.0 mg/mL, 46.76 % at 1.5 mg/mL, 41.65 % at 2.0 mg/mL and 29.67 % at 2.5 mg/mL. Similarly, the RSA was also seen to be highest at 0.5 mg/mL (91.42 %) and as the chitosan concentration increased, the scavenging activity decreased. The RSA at 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL and 2.5 mg/mL chitosan concentrations was found to be 84 %, 79.78 %, 60.11 % and 46.53 % respectively. On the other hand, the iron chelating activity was found to be maximum at 1.0 mg/mL (84.57 mg/mL) chitosan concentration, followed by 1.5 mg/mL (75.12 mg/mL) and 0.5 mg/mL (64.08 mg/mL) of chitosan concentration. Thereafter, the iron chelating activity of the seedlings decreased at 2.0 mg/mL (58.07 mg/mL) and 2.5 mg/mL (35.92 mg/mL) of chitosan concentrations. The chitinase activity started increasing from 0.5 mg/mL of chitosan concentration till 1.0 mg/mL, then the activity started decreasing till 2.5 mg/mL of chitosan concentration. The chitinase activity was seen maximum at 1.0 mg/mL of chitosan concentration, followed by 1.5 mg/mL, 2.0 mg/mL, 0.5 mg/mL and 2.5 mg/mL at values 2.338 IU/mL, 1.988 IU/mL, 1.660 IU/mL, 1.442 IU/mL and 1.361 IU/mL, respectively.

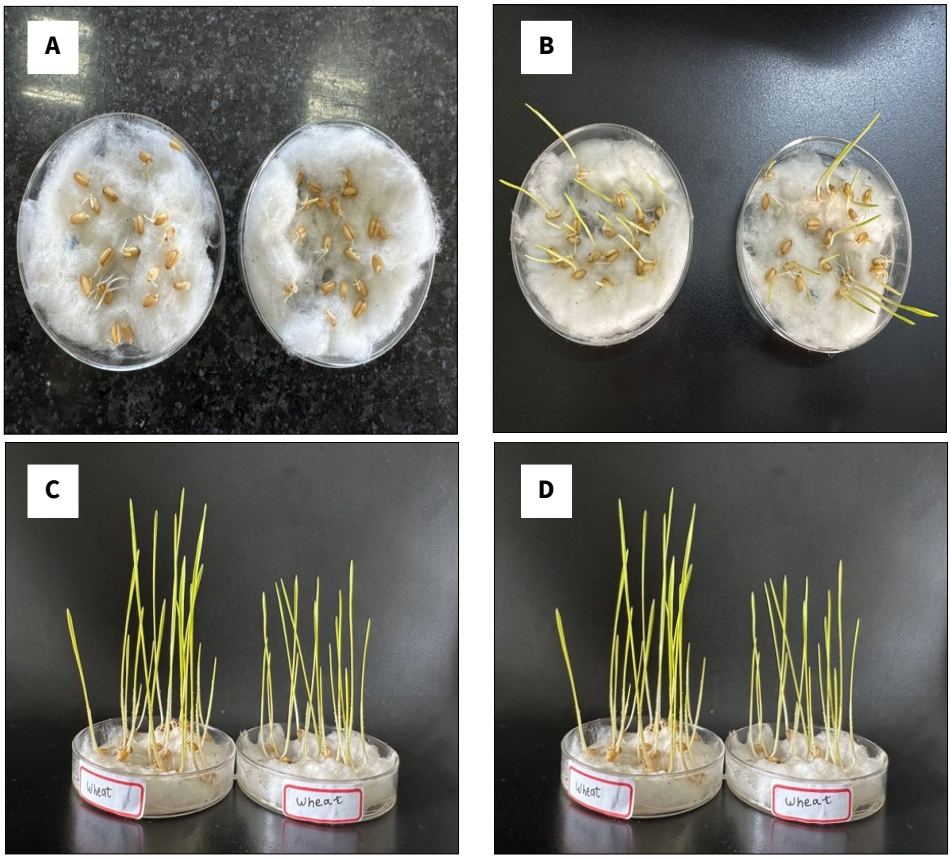

Fig. 1(A –D). Germination of wheat seeds under dark laboratory conditions on cotton. (A) Seeds at day 1; (B) Seedlings at day 7; (C&D) Seedlings at day 14.

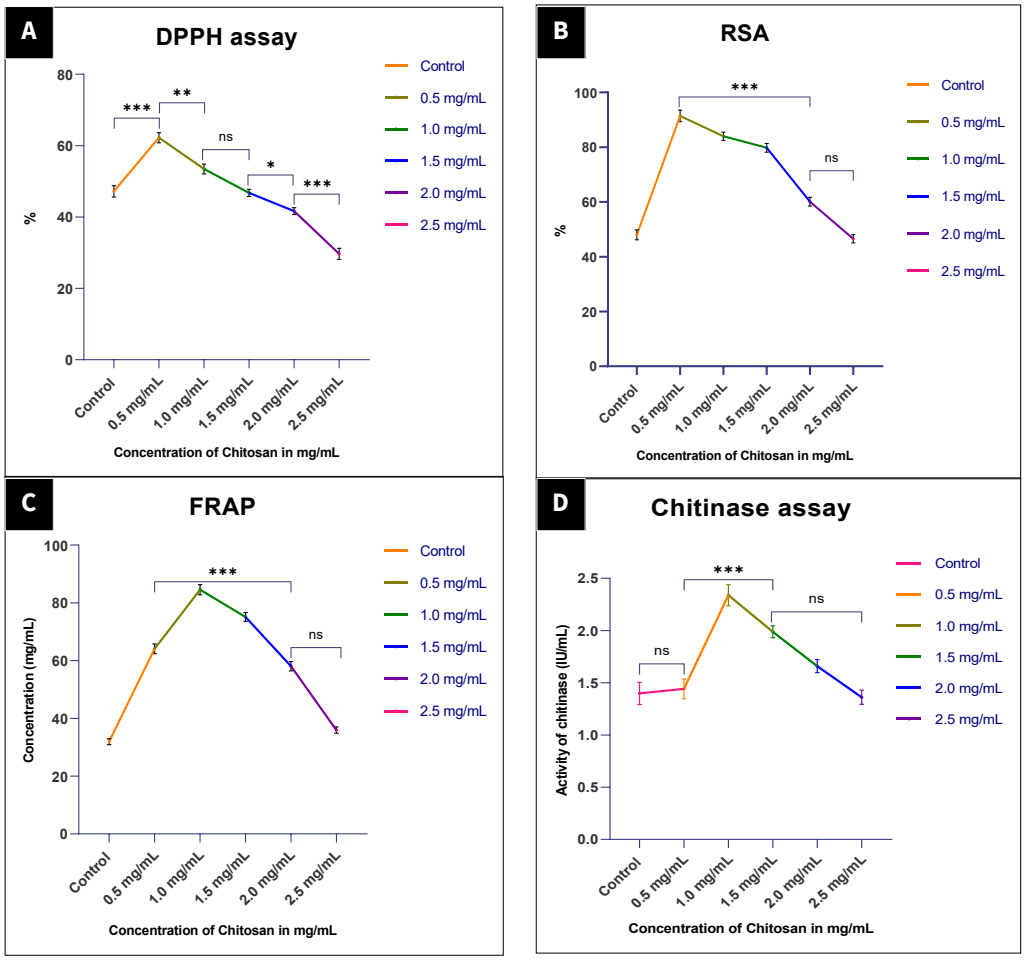

Fig. 2(A –D). Effect of chitosan treatment on antioxidant and defense-related activities in wheat seedlings. (A) DPPH radical scavenging activity; (B) Total RSA; (C) Iron chelating capacity; (D) Chitinase activity in wheat seedlings treated with chitosan (0.5–2.5 mg/mL). Values represent mean ± standard deviation (n = 3). Statistical significance relative to control is indicated as ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns represents non-significant.

Overall, the DPPH radical scavenging assay (Fig. 2A), activity increased significantly ($p < 0.001$) with chitosan treatment, reaching a maximum of 68.41 % ± 1.25 % at 1.0 mg/mL, which was markedly higher than the control (56.73 ± 0.98 %). A decline in activity was observed at concentrations above 1.0 mg/mL, with the lowest value recorded at 2.5 mg/mL (48.92 ± 1.57 %, $p < 0.001$ compared to 1.0 mg/mL) and the radical scavenging activity (Fig. 2B) exhibited a significant increase at 0.5 mg/mL (92.56 ± 1.42 %) and 1.0 mg/mL (91.84 ± 1.39 %) compared to the control (78.19 ± 1.12 %, $p < 0.001$). No significant difference (ns) was observed between 0.5 mg/mL and 1.0 mg/mL, whereas a sharp decline occurred at 1.5 mg/mL (73.65 ± 1.28 %) and higher concentrations. Similarly, the FRAP assay (Fig. 2C) followed a similar pattern, with the highest reducing capacity recorded at 1.0 mg/mL (88.72 ± 1.21 mg/mL equivalents), which was significantly greater ($p < 0.001$) than the control (59.48 ± 1.35 mg/mL equivalents). Beyond this concentration, FRAP values declined progressively. However, for chitinase activity (Fig. 2D), there was no significant change between the control (1.62 ± 0.05 U/mL) and 0.5 mg/mL treatment (1.74 ± 0.06 U/mL). The maximum activity (2.34 ± 0.07 U/mL, $p < 0.001$) was recorded at 1.0 mg/mL, after which enzyme activity decreased gradually, with no significant differences among 1.5 mg/mL, 2.0 mg/mL and 2.5 mg/mL treatments.

These results reveal a concentration-dependent response to chitosan, with 1.0 mg/mL consistently yielding the highest antioxidant and defense enzyme activities. Higher concentrations resulted in diminished responses, suggesting a threshold for optimal elicitation beyond which metabolic inhibition or stress-associated feedback regulation may occur.

Phenylalanine ammonia-lyase (PAL) assay

Fig. 3(A –D) shows the time-course response of PAL activity in wheat seedlings treated with various concentrations of chitosan (1 –4 mg/mL). Different concentrations of chitosan (1 mg/mL, 2 mg/mL, 3 mg/mL and 4 mg/mL) were exposed to the 14-day-old wheat seedlings and then, the PAL assay was carried out. Here, the applied elicitor had a positive effect on the enzyme activity in wheat seedlings. It was found that in 1 mg/mL of chitosan concentrations, peak activity of the PAL assay was seen at 48 hr (4.847 nM/min/gm ± 0.23), after that the activity of PAL started decreasing. Similarly, in 2 mg/mL of chitosan concentrations, the peak activity of the PAL assay was seen at 48 hr (7.077 nM/min/gm ± 0.19), after which the activity of PAL started decreasing. On the contrary, in 3 mg/mL of chitosan concentration, the peak activity was seen at 24 hr (4.022 nM/min/gm ± 0.17), after that, the activity started decreasing. Similarly, in the chitosan concentration- 4 mg/mL, the highest activity was seen at 24 hr (5.067 nM/min/gm ± 0.16), after which the activity started decreasing.

Overall, for 1 mg/mL chitosan (Fig. 3A), PAL activity increased significantly ($p < 0.001$) from the initial value of 2.14 ± 0.08 mM/min/g fresh weight (FW) at 0 hr to 4.73 ± 0.12 mM/min/g FW at 24 hr. The activity remained high at 48 hr (4.92 ± 0.11 mM/min/g FW, $p < 0.05$ compared to 24 hr) before declining to 3.56 ± 0.10 mM/min/g FW at 72 hr. In seedlings treated with 2 mg/mL chitosan (Fig. 3B), PAL activity rose moderately between 0 hr (5.92 ± 0.15 mM/min/g FW) and 24 hr (6.23 ± 0.14 mM/min/g FW, $p < 0.05$), followed by a sharp and highly significant ($p < 0.001$) increase at 48 hr (7.21 ± 0.13 mM/min/g FW). Activity decreased thereafter to 4.18 ± 0.12 mM/min/g FW at 72 hr. At 3 mg/mL chitosan (Fig. 3C), PAL activity increased significantly from 3.21 ± 0.09 mM/min/g FW at 0 hr to 4.35 ± 0.10 mM/min/g FW at 24 hr ($p < 0.001$), with no significant change at 48 hr (4.28 ± 0.08 mM/min/g FW). By 72 hr, activity dropped markedly ($p < 0.001$) to 1.94 ± 0.07 mM/min/g FW. In the 4 mg/mL treatment (Fig. 3D), PAL activity rose significantly ($p < 0.001$) from 3.26 ± 0.09 mM/min/g FW at 0 hr to 5.43 ± 0.12 mM/min/g FW at 24 hr. A gradual decline was observed at 48 hr (4.68 ± 0.11 mM/min/g FW, $p < 0.05$ compared to 24 hr) and 72 hr (2.67 ± 0.09 mM/min/g FW, $p < 0.01$ compared to 48 hr).

To summarize, chitosan treatments induced a time-dependent response in PAL activity, with early stimulation (24-48 hr) followed by a decline at 72 hr. The magnitude and timing of maximum PAL activation varied with elicitor concentration, with 2 mg/mL at 48 hr showing the highest overall activity. These results suggest that chitosan induces a transient upregulation of phenylpropanoid metabolism, likely linked to the activation of plant defense responses.

β-1,3 glucanase activity

Fig. 4(A-D) depicts the temporal changes in β-1,3-glucanase activity in wheat seedlings elicited across different concentrations of chitosan (1-4 mg/mL). Different concentrations of chitosan (1 mg/mL, 2 mg/mL, 3 mg/mL and 4 mg/mL) were exposed to the 14-day-old wheat seedlings and then, the β-1,3-glucanase assay was carried out. It was found that in 1 mg/mL of chitosan concentration, peak activity of the enzyme was seen at 24 hr (4835 IU/mg ± 197.9), after that, the enzyme activity started decreasing. However, in 2 mg/mL of chitosan concentrations, the peak activity of the enzyme was seen at 48 hr (4463 IU/mg ± 76.87), after which the activity of PAL started decreasing. Similarly, in 3 mg/mL of chitosan concentration, the peak activity was seen at 24 hr (4567 IU/mg ± 102.7), after which the activity started decreasing. On the contrary, in the chitosan concentration- 4 mg/mL, the highest activity was seen at 48 hr (4581 IU/mg ± 119.6), after which the activity started decreasing.

Overall, it was seen that for 1 mg/mL chitosan (Fig. 4A), β-1,3-glucanase activity increased significantly ($p < 0.001$) from 2874 ± 95 U/mg at 0 hr to 4562 ± 104 U/mg at 24 hr. The activity remained statistically unchanged between 24 hr (4562 ± 104 U/mg) and 48 hr (4498 ± 112 U/mg, ns), before declining to 3216 ± 98 U/mg at 72 hr. At 2 mg/mL chitosan (Fig. 4B), activity rose progressively from 2186 ± 88 U/mg at 0 hr to a maximum of 5124 ± 107 U/mg at 48 hr ($p < 0.001$), followed by a slight decrease to 4622 ± 101 U/mg at 72 hr. The 3 mg/mL treatment (Fig. 4C) also exhibited a significant increase from 2764 ± 92 U/mg at 0 hr to 4672 ± 108 U/mg at 24 hr ($p < 0.001$), with a further rise to 4982 ± 111 U/mg at 48 hr ($p < 0.05$). A pronounced decrease was recorded at 72 hr (3128 ± 96 U/mg). In the 4 mg/mL group (Fig. 4D), β-1,3-glucanase activity rose from 2986 ± 94 U/mg at 0 hr to 3874 ± 102 U/mg at 24 hr ($p < 0.05$), peaking at 4928 ± 110 U/mg at 48 hr ($p < 0.001$). A moderate reduction occurred at 72 hr (4102 ± 98 U/mg).

To summarize, chitosan treatments triggered a marked induction of β-1,3-glucanase activity, with the highest levels generally observed between 24-48 hr, depending on concentration. The strongest induction was recorded at 2 mg/mL after 48 hr. The subsequent decline in activity by 72 hr suggests transient activation of pathogen-related (PR) defense proteins, consistent with elicitor-induced early defense signalling in plants.

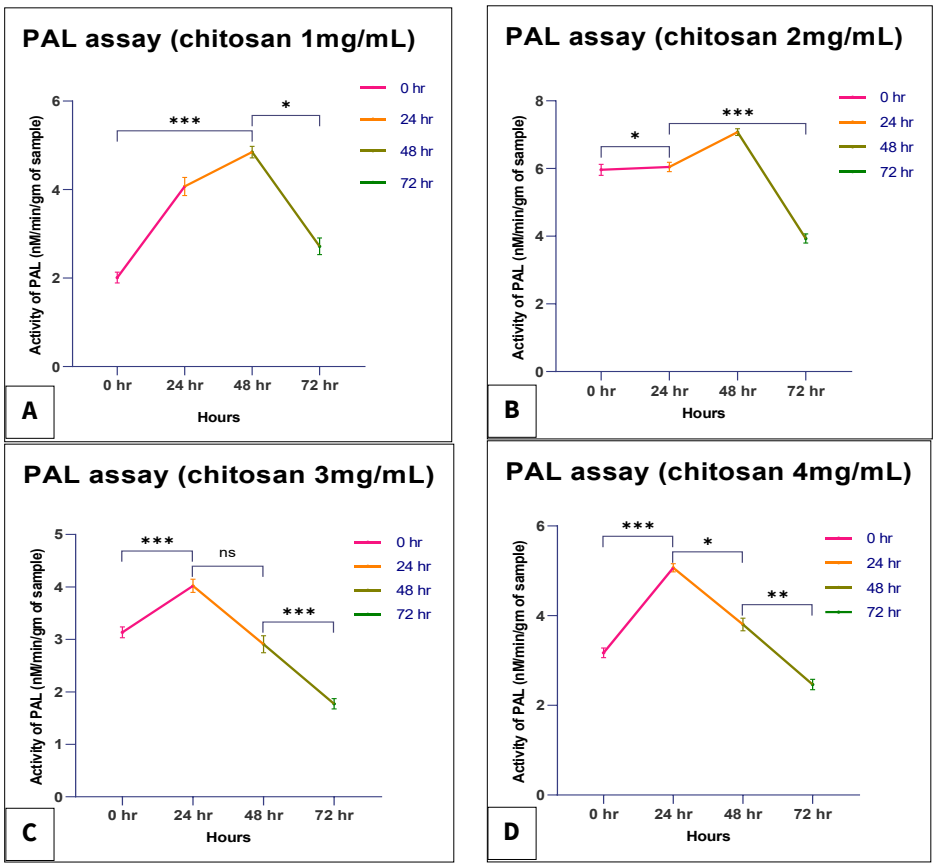

Fig. 3(A –D). Effect of chitosan elicitation on PAL activity in wheat seedlings. (A) 1 mg/mL chitosan; (B) 2 mg/mL chitosan. (C) 3 mg/mL chitosan; (D) 4 mg/mL chitosan treatments. Data are presented as mean ± standard deviation (n = 3). Statistical significance relative to control is indicated as ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns represents non-significant.

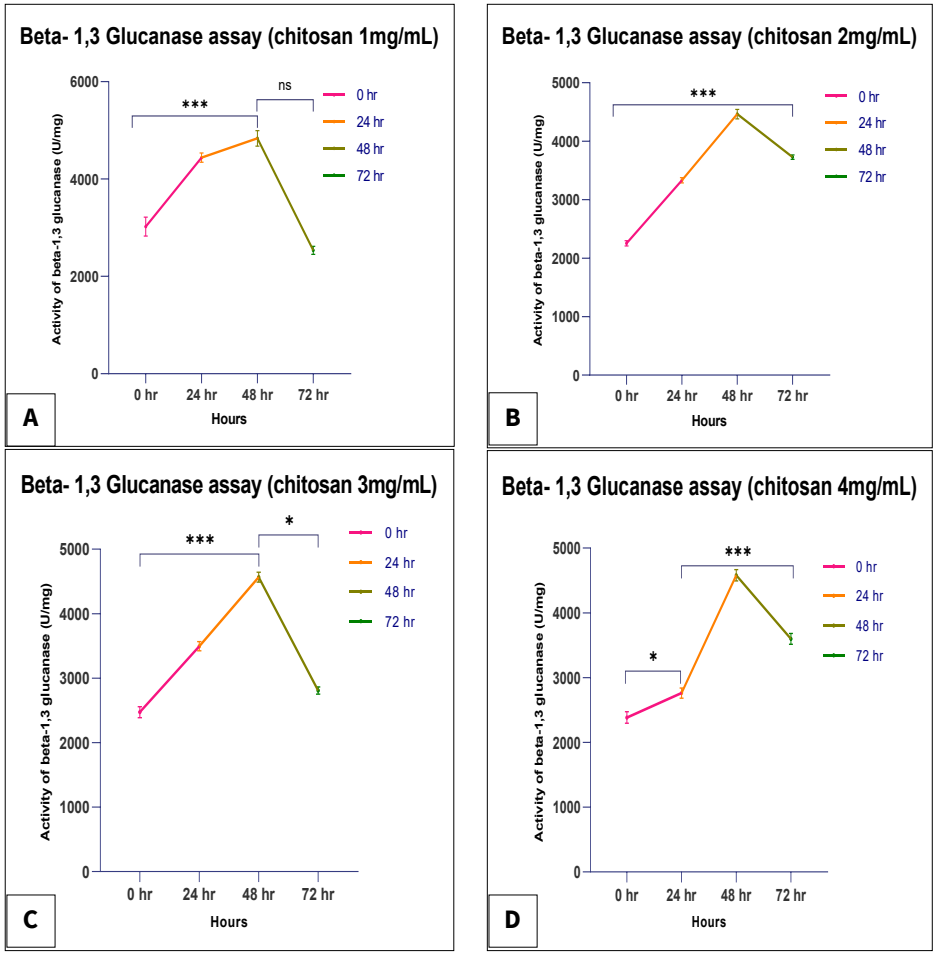

Fig. 4(A–D). Effect of chitosan elicitation on β-1,3-glucanase activity in wheat seedlings. (A) 1 mg/mL chitosan; (B) 2 mg/mL chitosan; (C) 3 mg/mL chitosan; (D) 4 mg/mL chitosan treatments. Data are presented as mean ± standard deviation (n = 3). Statistical significance relative to control is indicated as ***$p < 0.001$, **$p < 0.01$, and ns represents non-significant.

Discussion

Generation of reactive oxygen species (ROS) and antioxidant defense mechanisms

Under stress conditions, plants generate reactive oxygen species such as free radicals, superoxides and hydrogen peroxide (H_2O_2), leading to oxidative damage (32). The antioxidant potential of plants is commonly evaluated using assays such as 2,2-diphenyl-1-picrylhydrazyl and ferric reducing antioxidant power. Application of chitosan has been reported to modulate the antioxidant defense system in several plant species by enhancing both enzymatic and non-enzymatic components.

Effect of chitosan on growth and physiological attributes

Foliar application of chitosan solution before potting has been shown to enhance vegetative parameters, including the number of leaves, flowers, shoots and corms, as well as increasing chlorophyll content in *Freesia* (33). Similarly, chitosan treatments have improved plant growth, leaf morphology, stem and root development and overall plant vigour, with effects largely dependent on concentration, dosage and mode of application. At a concentration of 5 mg/L, chitosan promoted the accumulation of several secondary metabolites and enhanced antioxidant and anti-inflammatory activities (35). The combined use of chitosan and calcium carbonate ($CaCO_3$) further improved plant growth and productivity by increasing photosynthetic pigments, indole-3-acetic acid (IAA), soluble sugars, proline, phenolics and the activities of key antioxidant enzymes. Treated wheat plants also showed improved grain quality with elevated protein, carbohydrate, flavonoid and antioxidant contents (36).

Chitosan-mediated enhancement of phenolic and antioxidant compounds

Phenolic compounds are central to the antioxidant defense system in plants. In wheat, which is naturally rich in vitamin E and phenolic acids such as ferulic, caffeic, vanillic and *p*-coumaric acids, chitosan application has been shown to enhance the synthesis of these compounds (34). Studies in barley demonstrated that 1.0 % chitosan treatment significantly increased total phenolics and flavonoids, thereby improving the antioxidant and antimicrobial potential of sprouts within 24 hours of treatment (37). Furthermore, foliar chitosan application in wheat significantly improved grain yield and nutrient content under sandy soil conditions, with 100 mg L^{-1} treatment being most effective (38). Chitosan thus functions as a biostimulant, capable of enhancing both quantitative and qualitative parameters in cereal crops.

Chitosan-induced stress tolerance and antioxidant enzyme activation

Chitosan application mitigates oxidative stress by activating antioxidant machinery in plants exposed to abiotic stress, particularly salinity. In salt-stressed *Moringa* plants, chitosan improved growth, photosynthetic pigments and the activities of enzymatic antioxidants such as catalase, superoxide dismutase and ascorbate peroxidase (APX), as well as non-enzymatic antioxidants like carbohydrates, proline and phenols (41). In another study, accumulation of H_2O_2 was observed following both chitosan (CS) and chitosan nanoparticle (NP) treatments at 24 hours post-inoculation, with levels peaking at 72 hr (42). High concentrations of CS and NPs induced tissue damage, suggesting that chitosan and its nanoparticle forms elicit strong oxidative responses that may be harnessed for enhanced crop protection.

Modulation of phytochemical composition by chitosan

In *Cannabis sativa* L., treatment with chitosan at 50 mg/L and 250 mg/L significantly correlated with enhanced accumulation of flavonoids and tocopherols. Statistical analyses revealed substantial increases in total phenolic content (TPC; +36 % to +69 %), α-tocopherol (+45 % to +75 %), β+γ-tocopherol (+35 % to +82 %) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (+12 % to +28 %). Moreover, 50 mg L^{-1} chitosan treatment increased vitexin (+17 % to +20 %), orientin (+20 % to +30 %) and total flavonoid content (TFC; +12 % to +27 %) (43).

Role of chitosan in enhancing plant defense and hormonal regulation

In *Solanum tuberosum* infected with Potato virus Y (PVY), foliar chitosan application significantly improved physiological and biochemical parameters. Enhanced photosynthetic efficiency, chlorophyll content and osmoprotectant levels (glycine betaine, proline and soluble sugars) were observed. Chitosan also increased non-enzymatic antioxidants (glutathione, phenolics and ascorbic acid) and enzymatic antioxidants such as peroxidase, SOD, lipoxygenase, glutathione reductase (GR), CAT, β-1,3-glucanase and APX. Additionally, elevated phytohormone levels-including gibberellic acid (GA_3), IAA, jasmonic acid (JA) and salicylic acid (SA) and enhanced nutrient uptake (P, N, K) were reported (43). Phenylalanine ammonia-lyase, a key enzyme of the phenylpropanoid pathway, plays a central role in the synthesis of phenolic compounds, lignin, flavonoids and phytoalexins, which are critical in plant defense mechanisms.

Influence of chitosan on enzymatic activity and phenolic biosynthesis

Studies have shown that chitosan and fungal inoculation modify the activity of guaiacol peroxidase, polyphenol oxidase, PAL and APX. Variation in PAL activity at 2 days post-inoculation (DPI) was attributed to chitosan-induced responses in *Triticum aestivum* cv. Simeto and cv. Creso, with enhanced phenolic and antioxidant activity in both cultivars (44). Foliar application of salicylic acid-chitosan nanoparticles in wheat increased the activity of SOD, CAT, POD and polyphenol oxidase, while maintaining ROS, malondialdehyde and proline homeostasis (45). Further studies demonstrated that oligomers of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) with a degree of polymerization ≥7 induced POD activity, while partially N-acetylated chitosans activated both PAL and POD. Increasing chitosan concentrations enhanced ferulic acid accumulation and lignin synthesis, consistent with increased levels of phenolic acids such as p-coumaric, caffeic, chlorogenic and gallic acids (46).

Chitosan-induced modifications in cellular structure and redox regulation

In *Ceratocystis fimbriata*-infected sweet potato, chitosan treatment reduced fungal development and enhanced PAL and SOD activity without increasing MDA accumulation (47). Similarly, the combined application of nano-chitosan (NC), nano-silicon (NS) and nano-chitosan-encapsulated nano-silicon fertilizer (NC-NS) significantly improved antioxidant enzyme activities and redox homeostasis by regulating H_2O_2 levels in both leaves and roots

(48). In *Ocimum basilicum* L., chitosan elicitation substantially increased phenolic and terpenic compounds, with rosmarinic acid (RA) and eugenol levels rising by 2.5-fold and 2-fold, respectively, after 0.1-0.5 % chitosan treatment. DPPH radical scavenging activity increased by 3.5-fold while PAL activity increased 32-fold following treatment, accompanied by a 17 % increase in plant weight and 12 % increase in height (49).

Regulation of reactive oxygen species signalling and gene expression by chitosan

Chitosan treatments influence enzymatic activities related to fruit ripening and stress regulation. At 20 °C, chitosan limited the increase in soluble solids and total phenolics, while enhancing phenolic enzyme activity at 0 °C storage (50). An increase in RSA, FRAP and DPPH values up to optimal chitosan concentrations, followed by a decline at higher doses, suggests that chitosan activates specific defense-related genes rather than inducing generalized stress (51). Recognition of chitosan through pattern-recognition receptors such as chitin elicitor-binding protein and chitin elicitor receptor kinase 1 triggers N-acetylglucosamine-mediated signalling via mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs). This cascade activates transcription factors like WRKY, AP2/ERF and MYB (52), which regulate genes encoding SOD, CAT and APX- key antioxidant enzymes (53).

Integrative role of chitosan in antioxidant and phenylpropanoid pathways

The elevated RSA and FRAP values observed at 1.0 mg/mL chitosan reflect the upregulation of antioxidant enzymes. PAL assay data further confirm chitosan-mediated modulation of the phenylpropanoid pathway, which synthesizes lignin, flavonoids and cinnamic acid derivatives. The observed rise in PAL activity at 24 and 48 hr suggests synchronized activation of transcription factors such as MYB and NAC, promoting phenolic accumulation and antioxidant capacity (54). Transcriptomic studies in cereals such as wheat and rice have shown chitosan-induced upregulation of genes in the glutathione metabolism pathway, facilitating detoxification of lipid peroxidation products and maintaining cellular redox balance (55). Elevated FRAP values in the current study are consistent with enhanced glutathione activity and ferric ion reduction capacity. However, excessive ROS accumulation under higher chitosan concentrations leads to oxidative damage and downregulation of repair-associated genes (56).

Overall, the findings demonstrate that chitosan modulates both enzymatic and transcriptional responses by initiating ROS signalling cascades, activating stress-responsive transcription factors and upregulating genes associated with antioxidant, glutathione and phenylpropanoid pathways. The coordinated activation of these defense mechanisms explains the peak in antioxidant and defense enzyme activities observed up to 48 hr post-elicitation, highlighting chitosan's role as an efficient elicitor in enhancing plant secondary metabolism and stress resilience.

Conclusion

In conclusion, this study confirms that the chemical stress in plants induces adaptive defense mechanisms such as the secretion of non-enzymatic and enzymatic antioxidants. The wheat seedlings led to a significant increase in antioxidant activities and enzymatic activities such as PAL activity, chitinase activity and beta-1, 3-glucanase activity. Moreover, the results indicate that chitosan is a positive bioeffector that exerts a noteworthy effect on the growth and development of the plant. These changes might be further due to phenolic accumulation and activation of a few antioxidant enzymes. These findings suggest that chitosan can be used as a representation of a sustainable approach to help plants fight stressful conditions. The further prospects of this study can be applied to varying oligomer sizes of chitosan, since it might have a direct and indirect effect on the biological activity of the crop. The results obtained could give insight into breeding programs involving the selection of new stress-tolerant wheat genotypes. The study could be extended to field-grown plants to understand the comparative mechanism of stress response under field conditions.

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Authors' contributions

AS and PM[1] carried out the experimental assays. PM[2] and AD assisted in carrying out the experimental assays. MR helped in participated in the design and statistical analysis, drafted the manuscript. KM conceived of the study and participated in its design and coordination, edited the final draft of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflict of interest to declare.

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