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Chitosan alters protein of lupine (*Lupinus termis* L.) plant

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This study aimed to examine the impact of different concentrations of chitosan (0, 25, 50, 100, 150, 200, 250, 300, and 400 mg.L^{-1}) on the protein profile of lupine plants over a 45-day cultivation period. A pot experiment was conducted to grow lupine seeds treated with chitosan. After 45 days, the leaves were extracted, and protein banding patterns were analyzed using the SDS-PAGE technique. Protein number and protein intensity were modified about the corresponding control. Among the 15 protein bands, the highest protein weight was 169 kDs, whereas the lowest protein weighed 11 kDa. The 19 kDa protein, known as pathogenesis-induced protein (PR-10), was identified in response to 250, 300, and 400 mg.L^{-1}. Furthermore, there was a significant accumulation of γ-conglutins and δ-conglutins, weighing 12 and 13 kDa, respectively. The presence of high chitosan concentrations resulted in the absence of the 23 kDa structural protein. Chitosan modulates protein structure to promote growth and enhance defense against pathogens and environmental stress. We suggest using chitosan for agricultural lands that have been adversely affected by the overaccumulation of chemical fertilizers.

Keywords: Antioxidant activity, correlation matrix, germination, lupine, principal components analysis, survival percentage

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INTRODUCTION

Maintaining plant vigor throughout the growth and development stages is a complex issue, particularly for crops that are often contaminated with a wide range of chemical fertilizers. Researchers have developed chitosan and utilized it as a natural crop fertilizer. Chitosan is the cationic biopolymer derived from the deacetylation of chitin, the exoskeleton of marine organisms, fungi, invertebrates, insects, and microorganisms (Kumar, 2000; Stasinska-Jakubas & Hawrylak-Nowak, 2022). Chitosan has been widely documented in numerous publications as an organic, fast, degradable biostimulant, antitranspirant agent, bio-microbicide, global fertilizer, climatic change suppressor, and effective anti-stress agent (Hidangmayum et al., 2019; Kanawi, 2021; Drwish et al., 2023; Abed & Taha, 2024). In addition, chitosan has been reported as a soil remediator, serves as a crop fertilizer by facilitating the absorption of water and nutrients by plants (Kalia et al., 2020), a growth regulator, and a structure manager (Faqir & Chai, 2022). Moreover, chitosan plays a crucial role in the agricultural sector by enhancing the endurance of crop plants. It achieves this by binding to cellular receptors and releasing signaling molecules, including antioxidants, nitrogen oxides, calcium, catalase, monodehydroascorbate reductase, glutathione peroxidase, and growth promoters. These molecules facilitate a range of physiological processes in crop plants to resist harsh environmental conditions (Stasinska-Jakubas & Hawrylak-Nowak, 2022, Wang et al., 2020). Chitosan is a potent antifungal polymer that interacts with negatively charged molecules on the fungal cell surface, resulting in the disruption of cell wall intactness and leakage of cellular components to the outside. Chitosan stimulates the plant's innate immune system in the form of biosynthesis of secondary metabolites (Muthu et al., 2021), thereby improving plant responses to pathogen attacks and all types of stress (Pusztahelyi, 2018). Chitosan is likely to hinder soil-borne pathogens by stimulating root secretion into the soil (Suarez-Fernandez et al., 2020). These substances bind to toxins produced by the pathogens, resulting in a reduction in the opening of stomata and the development of structural changes such as lignification and mechanical barriers. These changes help to decrease plant conductance under adverse environmental conditions (Wang et al., 2020).

Lupine (*Lupinus termis* L.) is a member of the Fabaceae family and is named for its ability to produce *in situ* alkaloids, including lupanine, that are used to repel pathogens and animals (Cook et al., 2009). The growth-assisting proteins of lupine seeds are known as "conglutins" and are associated with seed vigor. These proteins provide both medicinal and nutritional benefits (Foley et al., 2017). Conglutins provide carbon, sulfur, nitrogen, and energy to growing plants. They are grouped into four families: α, β, γ, and δ conglutins (Duranti et al., 2008). The α and β conglutins are storage proteins that undergo degradation during germination. Among these, β conglutin (16-79 kDa) is the predominant storage protein in most lupine cultivars (Duranti et al., 2008). For γ-Conglutin, it is non-degradable at germination, whereas δ-conglutin is a vacuolar protein of low molecular weight, low

digestibility, and low water solubility. Additionally, it contains a range of enzymes and inhibitors (Ogura et al., 2013). The γ and δ- conglutin fractions (10-23 kDa.) exhibit an intensive appearance and the ability to accumulate twofold under cold temperatures (Dobiesz & Piotrowicz-Cieslak, 2017), indicating their role in stress conditions. Pathogenesis-related PR (PR) Proteins (15-19 kDa) are defensive proteins that are responsive to the PR-10 family, which includes fungi. In the vascular tissues of the lupine leaf, member proteins weighing 16.5 kDa have been detected and are marked as responsive to mosaic virus and abiotic stress, such as salicylic acid and UV radiation (Xie et al., 2010). PR proteins were first discovered in mosaic-virus-infected tobacco leaves (Va Loon et al., 1994) and subsequently observed in all plant species as regular proteins present in all organs. These proteins are localized either extra- or intracellular and categorized into 17 families based on their structure and activity (Christensen et al., 2002). These proteins are triggered in response to pathogen attacks and are involved in plant growth and development (Xu et al., 2014). Handschuh et al. (2007) discovered eight PR-10 genes in the leaves of white and yellow lupine. These genes have common characteristics such as the number of amino acids, low acidity, and protease tolerance (Liu & Ekramoddoullah, 2006). This study aims to promote the growth of lupine plants by utilizing chitosan to ensure the production of healthy and safe crops for consumers while also addressing the potential risks associated with chemical fertilizers. The potential effect of chitosan on lupine is examined by estimating the protein banding patterns using SDS-PAGE.

MATERIALS AND METHODS

Plant Materials

Lupine (*Lupinus termis* L.) seeds were provided by the Agricultural Research Center, Ministry of Agriculture, Dokki, Giza, Egypt.

Chemicals

The Lab chitosan $C_6H_{11}NO_{4.n}$ used in this study was industrialized for research and laboratory applications. The deacetylated chitosan (DA%:90-95) derived from shrimp exoskeletons was purchased from the National Research Center, Cairo, Egypt. The Acetic acid was obtained from Lab reagents and diluted at 5%, *v/v*. in distilled water and used as a chitosan solvent. Chitosan gradual concentrations (0.0, 25, 50, 100, 150, 200, 250, 300, and 400 mg. L^{-1}) were prepared in 5% acetic acid. The concentrations were selected based on the recommendations of previous literature reports. A.A treatment designated to chitosan free- acetic acid (5%).

Growth conditions

The pot experiment was conducted in two successive seasons (2020/2021 and 2021/2022) at the botanical garden of the Botany Department, Faculty of Science, Ain Shams University. Ten uniform L. termis L. seeds were sown in each plastic pot (16 × 6 cm LXW). The pots were filled with thin layers of beet-moss. The pots were categorized into ten groups. 1). The pots were irrigated with tap water "control". 2) The pots were irrigated with 5% acetic acid "A.A". 3) The pots were irrigated with 25 mg. L^{-1} chitosan "25". 4) The pots were irrigated with 50 mg.L^{-1} chitosan "50." 5) The pots were irrigated with 100 mg.L^{-1} chitosan "100" .6) The pots were irrigated with 150 mg.L^{-1} chitosan"150." 7) The pots were irrigated with 200 mg.L^{-1} chitosan "200." 8) The pots were irrigated with 250 mg.L^{-1} chitosan "250." 9) The pots were irrigated with 300 mg.L^{-1} chitosan "300" and 10) The pots were irrigated with 400 mg.L^{-1} chitosan "400". The experiment was conducted using a completely randomized design (CRD), with three pots allocated to each treatment. A total of 30 pots were kept under normal daylight and temperature conditions (38°/21°C). Seeds were left to grow in water for five days, and then two liters of water or chitosan concentration were used for 40 days of irrigation under a constant irrigation regime of 80% water holding capacity. At the end of the experiment, 45-day-old seedlings were photographed before being harvested. The seedlings were subsequently subjected to SDS-PAGE analysis to determine their protein banding patterns. The experiment was repeated five times, yielding consistent results. The statistical analysis was conducted using a one-way ANOVA at a significance level of 5%, with three replicates at each time point.

Methods

Protein banding patterns by SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was used to separate the protein subunits (Laemmli, 1970). SDS-PAGE was performed on stacking and separating gels using vertical slap gel electrophoresis (Bio-Rad, Hercules, CA, USA). The gel was adequately stained with Coomassie Brilliant Blue (G-250) and photographed. Data was analyzed using the Gel Documentation System (GDS). The Gel Analyzer 2010 software was

used to determine the percentage of band intensity, molecular weight, and mobility rate of each polypeptide in the protein samples about standard markers.

Statistical analysis

SDS-PAGE was repeated five times to obtain consistent data for the protein-banding pattern. Euclidean distance was used following data matrix scaling and standardization.

RESULTS AND DISCUSSION

Chitosan regulates the protein pool in lupine leaves

The image of the seedlings shows growth variations between chitosan-treated and untreated plants (Plate 1). The growth exhibited an upward trend beginning at a concentration of 150 mg.L-1 of chitosan, with the most optimal growth observed in lupine plants treated with a concentration of 200 mg.L-1 of chitosan. Analysis of the banding pattern of total extracted protein from 45-year-old lupine leaves demonstrated differences in the number and intensity of bands between the chitosan treatments and the control (Figure 1 & Table 1). To assess chitosan-induced changes in the protein pattern of the ten lanes (0.0, 25, 50, 100, 150, 200, 250, 300, and 400 $mg.L^{-1}$ chitosan and A), the gel was scanned using software programs.

The total number of protein bands per lane varied between 15 and 17, with molecular weights ranging from 169 to 11 kDa. The control bands are 169, 144, 102, 73, 64, 60, 48, 41, 37, 30, 28, 23, 19, 15, 13, and 11kDa. Chitosan was responsible for the expression of new protein bands and the disappearance of other proteins. Higher concentrations of chitosan typically resulted in the formation of new bands. For instance, the new protein band (83kDa) was expressed at 250 and 400 $mg.L^{-1}$, whereas the two intensive bands weighing 13 and 12 kDa were detected in the leaves of lupine treated with 250, 300 and 400 $mg.L^{-1}$ chitosan. A protein band with a molecular weight of 37 kDa was observed between 50 and 200 mg. The presence of L-1 chitosan was eliminated through treatment with acetic acid, as well as with other chitosan treatments, i.e., 100, 150, 250, 300, and 400 $mg.L^{-1}$. In addition, the 23 kDa protein band disappeared at 250, 300, and 400 $mg.L^{-1}$ chitosan, compared to the corresponding control. Analyzing the entire gel revealed 13 monolymorphic bands, 35% polymorphism, 0.8 band frequency, seven polymorphic with unique bands, and seven polymorphic without unique bands (Table 2). The electrophoretic analysis of protein banding patterns revealed the distribution of 22 bands in 45 d-old lupines, which is significantly higher than the 16 bands detected in 21 d-old lupine in a recent study (Beheiry et al., 2019). The presence of proteins with a molecular weight between 60-73 kD in both chitosan treatments and controls can be attributed to α- and β-conglutins. These proteins are known to be nutritive and typically have a molecular weight range of 53-74 kD, as reported in a recent study on narrow-leaflet lupine (Aiello et al., 2020). Chitosan modulates protein synthesis by stimulating hydrolytic enzymes required for the catabolic process of stored metabolites, which was previously discovered (Hameed et al., 2013). Therefore, the prominent protein bands with a weight of 12 and 13 kDa, which are observed in reaction to chitosan concentrations of 250, 300, and 400 $mg.L^{-1}$, are most likely γ- and δ- conglutin. These proteins have been previously identified as low molecular weight stress proteins (Dobiesz & Piotrowicz-Cieslak, 2017).

In this study, we investigated the effects of high concentrations of chitosan (250, 300, and 400 $mg.L^{-1}$) on the induction of ROS and the movement of γ-and δ-conglutinates from seeds to the leaves of lupine plants. We found that these high chitosan concentrations facilitated the upward movement of γ-and δ-conglutinates through the vascular system, resulting in their reappearance in lupine leaves as three distinct heavy polypeptide bands weighing 12 and 13 kDa. The weak growth appearance indicates that γ- and δ-conglutin are unable to enhance the vigor of lupine plants when exposed to high doses of chitosan (Dobiesz & Piotrowicz-Cieslak, 2017). Consequently, the inability to provide catabolic supplements to sprouted seedlings results in sluggish growth, reduced germination rate, and diminished plant survival, in addition to other effects of ROS and possible electrolyte leakage (EL) under high chitosan doses. Although PR-10 (15 and 19 kDa) was detected under normal conditions and with all chitosan treatments, the accumulation of 15 kDa and 19 kDa was significantly higher under the influence of chitosan treatments, as evidenced by their protein bands (Figure 2). This indicates that chitosan plays a role in stimulating pathogen defense mechanisms. Nevertheless, the defense mechanism proved inadequate to protect lupine growth in terms of lack of energy and nutrition expected from seeds and releasing of ROS and high EL and low membrane stability index under high chitosan concentrations (250, 300, and 400 $mg.L^{-1}$).

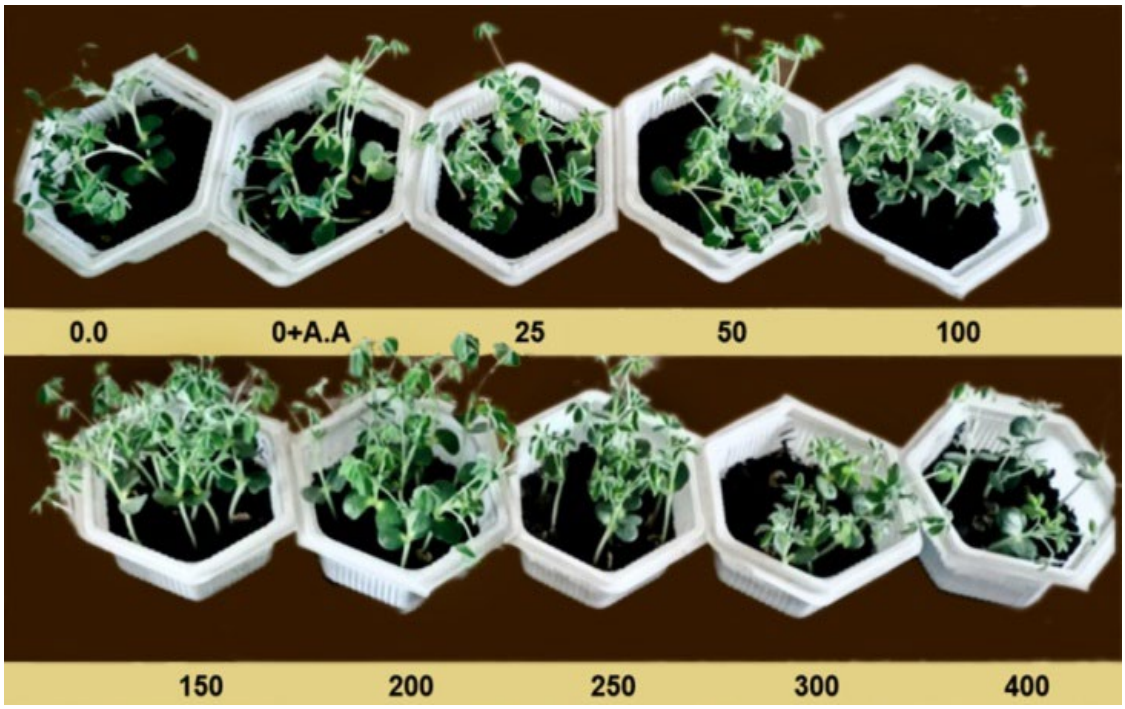

Plate 1. A photograph showing growth variations of 45-d old lupine plants irrigated using different concentrations of chitosan (0.0, 25, 50, 100, 150, 200, 250, 300, and 400 mg. L^{-1}) in addition to 5% acetic acid (A.A).

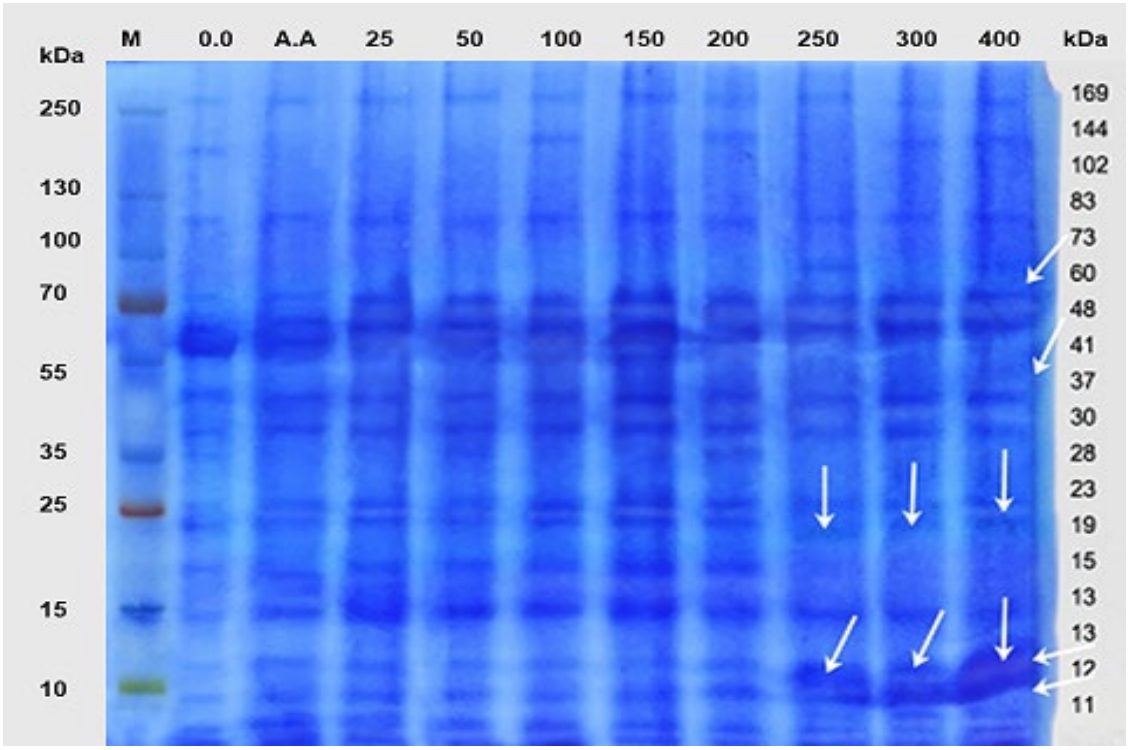

Figure 1 The SDS-PAGE electropherograms illustrate the banding pattern of total leaf proteins extracted from 45-d old lupine irrigated with different chitosan concentrations (0.0, 25, 50, 100, 150, 200, 250, 300 and 400 mg.L^{-1}) in addition to 5% acetic acid (A.A), as indicated in lane labeling. Marker, M; water control, 0.0; chitosan free- acetic acid, A.A. Separate arrows point to the place of band disappearance, and the three arrows below point to the intense appearance of three bands at 12 and 13 kDa with 250, 300, and 400 mg.L^{-1} chitosan.

Table 1. Chitosan treatments regulate protein patterns and frequencies in 45-d old lupine seedlings irrigated using different concentrations of chitosan (0.0, 25, 50, 100, 150, 200, 250, 300, and 400 mg. L^{-1}) in addition to 5% acetic acid (A.A). Green boxes view new bands, and gray boxes mark band disappearance.

Band No	Mwt kDa.	Chitosan mg.L^{-1}.										Freq.
		0.0	A.A	25	50	100	150	200	250	300	400	
1	169	1	1	1	1	1	1	1	1	1	1	1.0
2	144	1	0	0	0	1	0	1	0	1	1	0.5
3	102	1	1	1	1	1	1	1	1	1	1	1.0
4	83	0	0	0	0	0	0	0	1	0	1	0.2
5	73	1	1	1	1	1	1	1	1	1	1	1.0
6	64	1	1	1	1	1	1	1	1	1	1	1.0
7	60	1	1	1	1	1	1	1	1	1	1	1.0
8	48	1	1	1	1	1	1	1	1	1	1	1.0
9	41	1	1	1	1	1	1	1	1	1	1	1.0
10	37	1	0	0	1	0	0	1	0	0	0	0.3
11	30	1	1	1	1	1	1	1	1	1	1	1.0
12	28	1	1	1	1	1	1	1	1	1	1	1.0
13	23	1	1	1	1	1	1	1	0	0	0	0.7
14	19	1	1	1	1	1	1	1	1	1	1	1.0
15	15	1	1	1	1	1	1	1	1	1	1	1.0
16	13	0	0	0	0	0	0	0	1	1	1	0.3
17	13	1	1	1	1	1	1	1	0	0	0	0.7
18	12	0	0	0	0	0	0	0	1	1	1	0.3
19	11	1	1	1	1	1	1	1	1	1	1	1.0
20	11	1	1	1	1	1	1	1	1	1	1	1.0
Band no/lane	20	17	15	15	16	16	15	17	16	16	17	-
No. of new bands	-	-	0	0	0	0	0	0	3	2	3	-
No. of disappeared bands	-	-	1	1	0	1	1	0	2	3	2	-

Table 2. Polymorphism of SDS-PAGE extracted lupine proteins from grown seedlings with chitosan treatments.

Gel Polymorphism	
Monomorphic bands	13
Polymorphic without Unique.	7
Unique bands	0
Polymorphic with Unique.	7
Total number of bands	20
Polymorphism %.	35%
Mean of band frequency	0.8

Prior research has identified proteins in the lupine cortex and stem with a molecular weight ranging from 21 to 42 kD (Pinheiro et al., 2005). The disappearance of a protein band weighing 23 kDa at high chitosan doses (250, 300, and 400 mg.L^{-1}) highlights the deleterious effect of high chitosan concentrations on lupine structural proteins as well. The reduced growth observed with high chitosan doses can be attributed to the depletion of the cortex and stem proteins required for structural development and optimal growth. In agreement with previous findings, the expressed polypeptide weighing 48 kDa in lupine leaves was identified as an esterase isozyme, whereas the polypeptide weighing 30 kDa was identified as a catalase enzyme (Beheiry et al., 2019).

CONCLUSION

Chitosan regulates protein synthesis to enhance growth of lupine plants; the impact influenced by chitosan concentrations. PR-10 proteins associated with pathogenesis are expressed by low and medium doses of chitosan to protect lupine from air- and soil-borne pathogens and create growth conditions that are appropriate to safe culture. This study indicates that 200 mg. L-1 chitosan is a substitute for chemical fertilizers and contributes to the optimal growth of lupines.

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