ANNALI DI BOTANICA
Ann. Bot. (Roma), 2021, 11: 121–134
Journal homepage: http://annalidibotanica.uniroma1.it

THE EFFECTS OF CHITOSAN APPLICATION AGAINST ALUMINUM TOXICITY IN WHEAT (*TRITICUM AESTIVUM* L.) ROOTS

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(RECEIVED 12 JULY 2020; RECEIVED IN REVISED FORM 19 NOVEMBER 2020; ACCEPTED 30 NOVEMBER 2020)

ABSTRACT - Aluminum (Al) is the most common metallic element after oxygen and silicon, which makes up 8.1% of the earth's crust. If the acidity of the soil increases due to several environmental factors, Al is dissolved and transformed into a toxic form. In acidic soils, dissolved Al, usually present in concentrations ranging from 10 to 100 µM, is an important factor in inhibiting growth for plants. In the present study, we aimed to alleviate the Al toxicity by chitosan (CHT) application in wheat. Wheat (*Triticum aestivum* L.) seeds germinated for 96 h in different solutions. Distilled water was used as control. Toxicity was established using 100 µM $AlCl_3$ solution (pH 4.5). Roots were treated with 0.1, 0.25 and 0.5 mg/L CHT solutions to alleviate toxicity. Based on our results, while Al inhibited the elongation of wheat roots, an increase in root elongation was observed as a result of the CHT application. Al-induced lipid peroxidation, loss of membrane integrity, proline deposition, and antioxidant enzyme activities. The Al-damage was improved by CHT and Al-resistance rose with increasing antioxidant enzyme activity. As a result, the alleviation effects of CHT on Al-induced stress symptoms were found to be higher in lower concentrations (0.1 and 0.25 mg/L).

KEYWORDS: AL TOXICITY; ANTIOXIDANT ENZYME ACTIVITY; CHITOSAN; LIPID PEROXIDATION; ROOT; WHEAT.

INTRODUCTION

Aluminum (Al) toxicity is one of the abiotic stress factors that plants are frequently exposed to, especially in acidic soils. Aluminum is one of the most abundant minerals in the earth's crust and is found in the form of insoluble complex compounds (aluminum silicates or oxides) (Kochian, 1995). It becomes soluble in acidic soils with a pH below 5 by various factors such as climate and environmental pollution. Aluminum ions taken by plant roots inhibit plant growth and function in a short time (½ hour), even at micromolar (µm) concentrations (Jones & Kochian, 1995). The most important symptom of Al toxicity is inhibition of root growth (Hasenstein et al., 1988). The target of Al toxicity is the root tip, in which Al exposure causes inhibition of cell elongation and cell division, leading to root stunting accompanied by reduced water and nutrient uptake (Jones et al., 2006; Zhang et al., 2007; Bose et al., 2011). When Al negatively affects root development, it causes a regression in plant growth (Blarney et al., 2004; Ma, 2004). As a result of the regression, the yield and quality decrease in agronomic products.

Chemical methods that have been used for many years to reduce the economic losses during agricultural production have a negative effect on human health and ecological balance. Therefore, in recent years, researches about harmless environmental improvement methods have increased. One of the preferred natural conditioners as an alternative to chemical products is chitosan (CHT). CHT is obtained from the chitin deacetylation method, which is commonly found in the skeleton of shellfish such as crab, lobster, shrimp (Kumar, 2000). It is one of the naturally occurring compounds that have potential in agriculture regarding controlling plant diseases. Besides having antiviral, antibacterial, and antifungal properties, CHT is an effective agent in controlling and reducing the spread of diseases by promoting the defense system of plants. In addition to this, it has been started to be used in the agricultural

doi: 10.13133/2239-3129/16884

field as chelators to metal ions in its environment (water, soil, etc.) and prevents the ingestion of toxic effective metals by the plants (Vasconcelos, 2014; Malerba & Cerana, 2016). Despite many studies, the mechanism of CHT activation in plants has not been fully elucidated. The increase in detailed studies on CHT will help to obtain high yields of products with the use of CHT in agricultural fields. The application of CHT against abiotic stress in plants has also attracted attention recently. Li et al. (2016) obtained CHT improves drought resistance in white clover (*Trifolium repens* L.) by enhancing the accumulation of stress-protective metabolites. Zong et al. (2017) reported alleviation against the toxic effects of cadmium through foliar application of different molecular weight of CHT in rape (*Brassica rapa* L.). Basit et al. (2020) stated that the application of CHT had a positive impact on quality indices of tomato plants under water stress. Bakhoum et al. (2020) applied CHT to the sunflower plant under salinity stress. Researchers stated that CHT treatments did not only improve plant growth and productivity but also could enhance the resistance of salinity stress. Recently Hafez et al. (2020) proved the significance of CHT in alleviating the damaging impacts of drought on barley plants. Although CHT has been used as a protective material against drought, low pH, and cadmium stress, and to the best of our knowledge, there is no research on alleviation of Al toxicity with CHT application. In the present study, we aimed to improve the Al toxicity with CHT application in wheat which has been used as food worldwide.

Materials and Methods

Plant material and Al/CHT treatment

The wheat (*Triticum aestivum* L. cv Demir 2000) seeds were provided from the Republic of Turkey Ministry of Agriculture and Forestry Field Crops Central Research Institute (Ankara). Wheat seeds were sterilized with 1% sodium hypochlorite solution (5% available chlorine) for 10 min and rinsed several times in sterile distilled water. Then seeds were placed for germination in Petri dishes (15 cm) with Al (100 µM, pH 4.5) and different concentrations of CHT solutions (0.1, 0.25 and 0.5 mg/L) which were decided after optimization studies. 40 seeds in each glass dish were germinated for 96 h under plant growth room conditions (temperature of 23 ± 2 °C, relative humidity 45-50% and a light intensity of 5000 lux day/night: 16/8). Distilled water was used as a negative control group. While only CHT solutions were used as a positive control, Al and CHT solutions of different concentrations were applied together as alleviation groups. Each solution was refreshed every 24 h. Germinated seeds that reached 0.5-1 cm root elongation were used for analyses. Control and treated roots collected and stored at -80 °C until analysis.

Preparation of CHT and Al solutions

In this study, aluminum chloride hexahydrate ($AlCl_3.6H_2O$) (Emprove) and low molecular weight CHT were used (Sigma-Aldrich STBG9041).
Stock chitosan solution was prepared in 1% (v/v) acetic acid at 1% (w/v) concentration and diluted with water or $AlCl_3$ solution (100 µM) to give a final chitosan concentration of 0.1, 0.25 and 0.5 mg/L. All solutions were freshly prepared before application.

Measurement of growth

Germination rate (%) was calculated as (number of germinated wheat sprouts [> 1]/ total number of wheat seeds) x 100. For measurement of relative primary root length of 30 wheat sprouts were randomly sampled from each tray. The % root lengths were calculated according to Teskey & Hinckley (1981) (elongation of control-elongation of treatment group)/ (elongation of control) x 100.

Determination of lignin and callose deposition

To determine the lignin and callose accumulation, fresh roots after 96h were cut and rinsed in distilled water. To observe lignin accumulation, the method of staining with phloroglucinol + 25% HCl was used (Çakırlar et al., 2010). Fresh roots of the control and treatment groups were cut and taken on the slide. A few drops of phloroglucinol solution were dropped after 5 min photographed under a stereomicroscope (Leica brand EZ4 HD). The method of Kenrick & Knox (1985) was used to observe the accumulation of callose. Roots were stained with 0.1% aniline blue for an hour in the dark and rinsed in 0.1 M K_3PO_4 (pH 8). The roots were monitored at 330-385 nm with KAMERAM software, assisted by a KAMERAM fluorescent camera and an Olympus BX-51 fluorescence microscope.

Determination of Al ions

The hematoxylin staining method (Ownby, 1993) was used to determine Al ion uptake in the roots. After rinsing fresh wheat roots, 10 individuals from each group were stained with hematoxylin solution [0.2% hematoxylin and 0.02% potassium iodide, w/v] for 15 min in the dark. After rinsing the excess dye in distilled water for 10 min, equal length (10 mm) of root tips were excised and placed in glass tubes. The control and treated root tips were immersed in 1 N HCl solution for an hour. The supernatant was measured at 490 nm spectrophotometrically by IMPLEN P330.

Determination of loss of plasma membrane integrity

The loss of plasma membrane integrity was evaluated by Evans blue staining in control and treated roots (Schützendübel et al., 2001). The intact wheat roots were rinsed in running tap water and stained with 0.25% (w/v) Evans blue solution for 30 min. After rinsing the excess dye in distilled water for 10 min, 10 root tips of equal length (10 mm) were excised. The control and treated roots were homogenized in 1 mL of 1% (w/v) sodium dodecyl sulfate (SDS) solution and centrifuged at 13.500 g for 10 min. The supernatant was measured at 600 nm spectrophotometrically by IMPLEN P330.

Determination of lipid peroxidation

Lipid peroxidation was determined by the concentration of malondialdehyde (MDA) after reaction with thiobarbituric acid (TBA) (Cakmak & Horst 1991). The control and treated roots (0.2 g) were homogenized with 1 mL 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12.000 g for 20 min. Supernatants (250 µL) were mixed with 0.6% (w/v) TBA in 20% (w/v) TCA (1 mL) and boiled at 95 °C for 30 min. The mixture was cooled immediately on ice and centrifuged at 12.000 g for 10 min. The absorbance of the TBA-reactive substance was determined as TBA-MDA complex at 532 and 600 nm. The amount of MDA was calculated according to the formula below.

$$\mathrm{MDA} = \frac{\Delta A\,(532-600)}{1.56*10^5} \qquad \text{Eq. 1}$$

Determination of guaiacol peroxidase activity

Guaiacol peroxidase (GPX) activity was measured using the method of Birecka et al. (1973). 0.1 g roots were homogenized with extraction buffer (0.1 M PBS, pH 7.0) at +4 °C. The homogenates were centrifuged at 11.000 rpm for 25 min at +4 °C. The reaction mixture containing 1.5 mL substrate buffer (0.1 M PBS pH 5.8, 5 mM H_2O_2, 15 mM guaiacol) and 20 µl enzyme extract (supernatant) were measured immediately for 2 min at 470 nm. The absorbances were formulated as

$$\mathrm{GPX} = \frac{\Delta A}{\mathrm{gFWmin}} \qquad \text{Eq. 2}$$

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was evaluated by the method of Giannopolitis & Ries (1977). 0.1 g roots were homogenized with PBS (pH 7.0) including 0.1 mM EDTA, 1% Polyvinylpyrrolidone (PVP) (w/v) at +4 °C. The homogenates were centrifuged at 14.000 rpm for 20 min at +4 °C. Supernatant (3 µL) and 3 mL reaction mixture (0.1 M phosphate buffer pH 7, 2 M Na_2CO_3, 10 mM EDTA, 300 mM L-methionine, 7.5 mM NBT, 0.2 mM riboflavin) was kept under white fluorescent lamps (15 W) for 10 min in test tubes. The absorbance was measured at 560 nm. Then, the % inhibition values of the samples were determined according to the formula below.

$$\mathrm{Inhibition} = \frac{\text{Sample } \Delta A560 - \text{Blank } \Delta A560 * 100}{\text{Positive Control } \Delta A560 - \text{Blank } \Delta A560} \qquad \text{Eq. 3}$$

Determination of total protein content

The total protein content of control and treated roots was measured according to Bradford (1976). The roots were extracted in 0.1 M PBS (pH 7.7) with a chilled mortar and pestle with a ratio of 1 g/3 mL buffer. The homogenates were centrifuged at 12.000 rpm for 20 min at 4◦ C. The reaction mixture including Bradford reagent (5 mL) and supernatant (100 µL) was measured at 590 nm spectrophotometrically by IMPLEN P330. A standard curve was constituted with a series of dilutions of bovine serum albumin (BSA).

Determination of total proline content

Total proline content was determined by the method of Bates (1973). 0.1 g roots were homogenized in 10 mL sulfosalicylic acid (3% w/v) and centrifuged at 5.000 g for 10 min. The supernatant (2 mL) was mixed with equal quantities of glacial acetic acid-ninhydrin reagent, and boiled for 1 h at 100 ºC. The solution was mixed with 5 mL toluene on ice. After separation of solution layers, the toluene layer was carefully removed and placed in glass cuvettes, and absorption was determined at 520 nm. Proline concentrations were determined from a standard curve and calculated on a fresh weight basis. The results obtained, the amount of proline (µmol/g FW) was calculated according to the formula below.

$$\mathrm{Proline} = \frac{\text{Proline } \mu g/mL * 4}{115.5/(\frac{g}{5})} \qquad \text{Eq. 4}$$

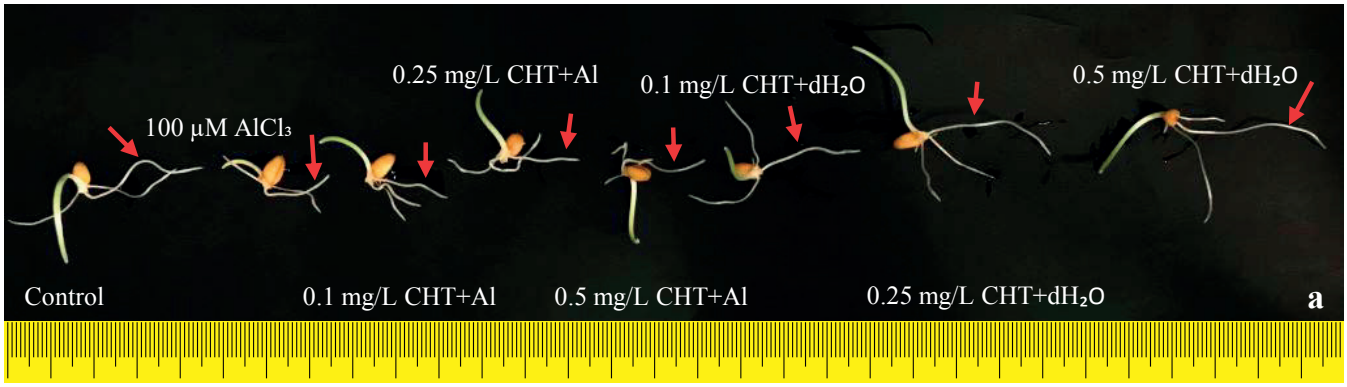
where FW is the fresh weight of roots, Prolin µg/mL is the amount of proline calculated according to the calibration curve, g is referred to as the sample weight, 115.5 is the molecular weight of proline.

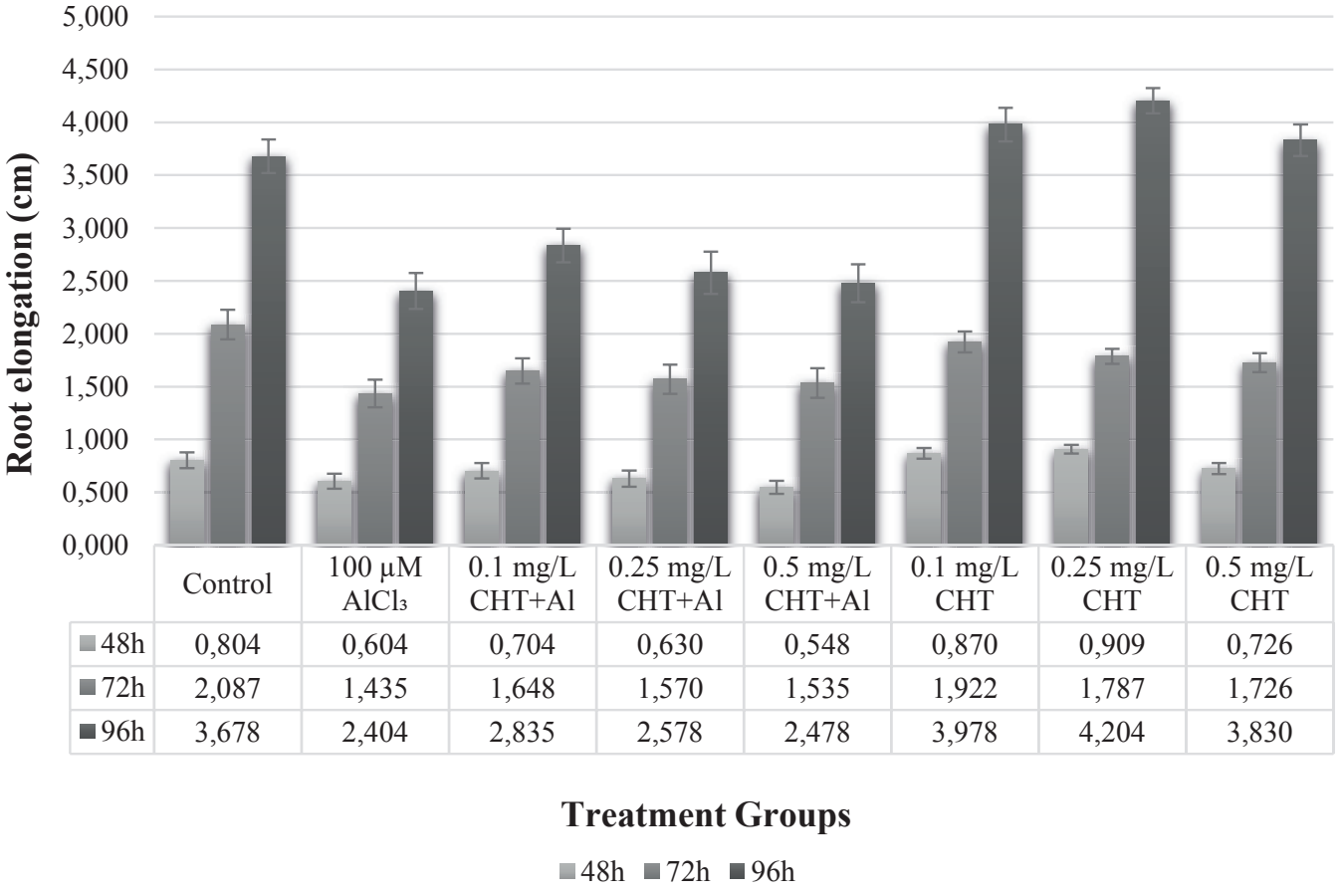
Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), (SPSS 16.0 software). The significance of the applications was designated at the $P < 0.05$ level using Tukey's test. All data presented are means ± Standard Deviation (SD). All of the analyses were assessed with three replicates.

RESULTS

To determine the alleviation effects of chitosan (CHT) on Al toxicity, morphological and biochemical analyses were performed in wheat roots after 96 h. According to our results, root elongation was inhibited by 34% after 100 µM Al treatment. However it was increased by 8% in 0.1 mg/L CHT, 14% in 0.25 mg/L CHT and 4% in 0.5 mg/L CHT. In alleviation groups, the root elongation increased by 17% in 0.1 mg/L CHT + Al, 7% in 0.25 mg/L CHT + Al and 3% in 0.5 mg/L CHT + Al in compare to Al application. Our results revealed that Al inhibited root elongation progressively, but CHT application ameliorated root growth especially in 0.1 mg/L CHT (Figure 1).





	Control	100 µM AlCl3	0.1 mg/L CHT+Al	0.25 mg/L CHT+Al	0.5 mg/L CHT+Al	0.1 mg/L CHT	0.25 mg/L CHT	0.5 mg/L CHT
48h	0,804	0,604	0,704	0,630	0,548	0,870	0,909	0,726
72h	2,087	1,435	1,648	1,570	1,535	1,922	1,787	1,726
96h	3,678	2,404	2,835	2,578	2,478	3,978	4,204	3,830

Figure 1. Root elongation of control and experimental groups in wheat. (a) Morphological view of wheat roots after 96 h (individuals in the picture present the average morphological state in each groups); (b) Root elongations data of means. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD. Statistics belong to control and application groups after 96 hours.

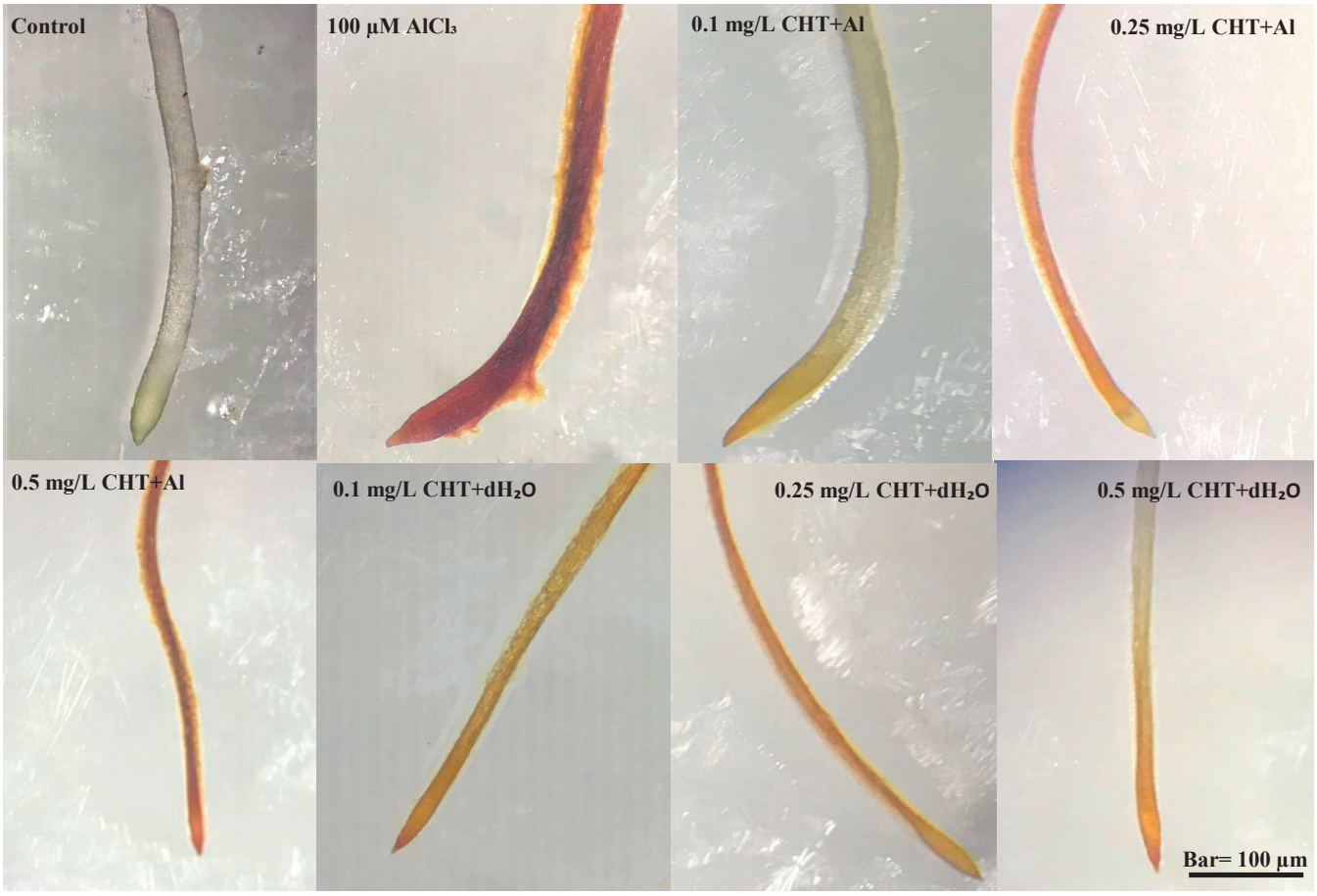


Figure 2. Lignin deposition of control and experimental groups in wheat at 96 h (Bar: 100 µm).

To correlate the effects of CHT on Al toxicity, some histochemical tests were performed. Lignin, which is a complex polymer of alcohols, was confirmed by acid phloroglucinol. Lignin accumulation was observed as dark red due to stress in Al treatment. It has been observed that lignin accumulation was visible in both positive control and Al-CHT groups, while the closest appearance was in 0.1 mg/L CHT + Al group compared to the control (Figure 2).

The formation of callose, which is one of the sensitive stress factors, was revealed with the fluorescent dye aniline blue in bright spots in the elongation and maturation zones of wheat roots. According to our results, there was no significant difference between control and positive control groups. Similarly no significant bright spots were observed in 0.1 and 0.5 mg/L CHT + Al, except for 0.25 mg/L CHT + Al. However, callose deposition was very significant in 100 µM Al (Figure 3).

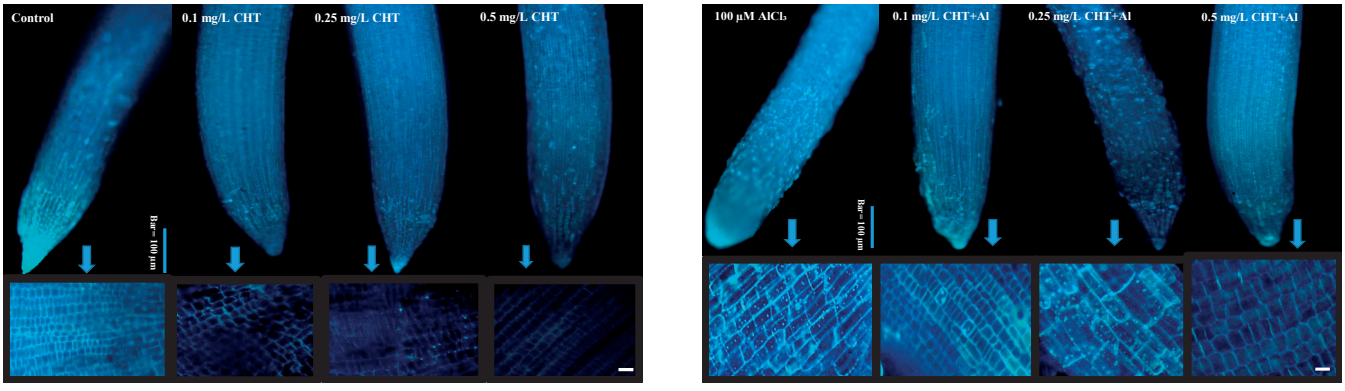


Figure 3. Callose deposition of control and treated wheat roots at 96 h (Bar: 100 µm).

Wheat roots were stained with hematoxylin to measure the Al uptake. According to our results, the amount of Al absorbed from the roots was decreased by 44% in 0.1 mg/L CHT + Al and by 10% in 0.25 mg/L CHT + Al in comparison to Al treatment. However, it was increased by 9% in 0.5 mg/L CHT + Al. Based on our results, 0.1 mg/L CHT was found to be very effective in reducing Al ion uptake (Figure 4).

The loss of plasma membrane integrity was determined by Evans blue assay. The uptake of dye was increased by 3.4 fold in Al concerning control. It was decreased by 19% in 0.1 mg/L CHT + Al and 51% in 0.25 mg/L CHT + Al and 36% in 0.5 mg/L CHT + Al with regard to Al treatment. Based on these results, 0.25 mg/L CHT was found to be very effective in reducing the loss of plasma membrane integrity (Figure 5).

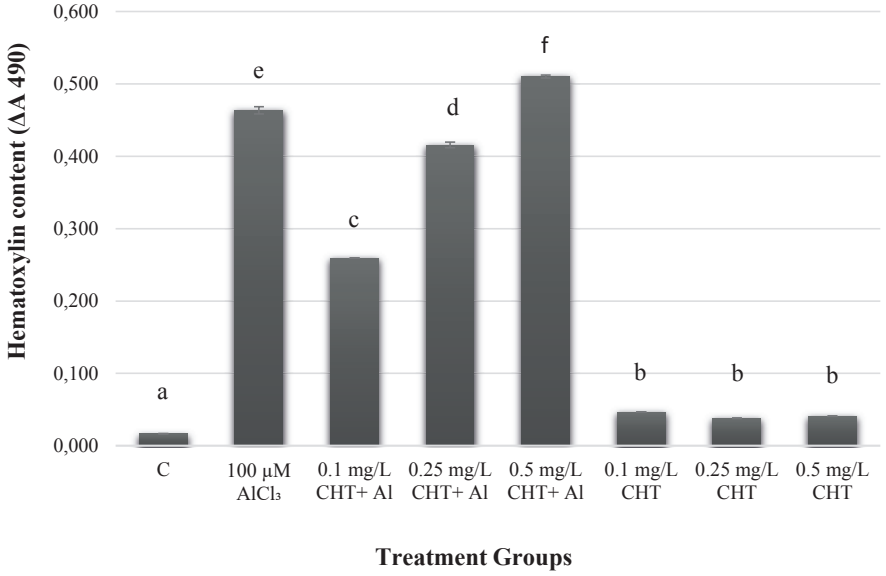


Figure 4. Aluminum ion uptake of control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

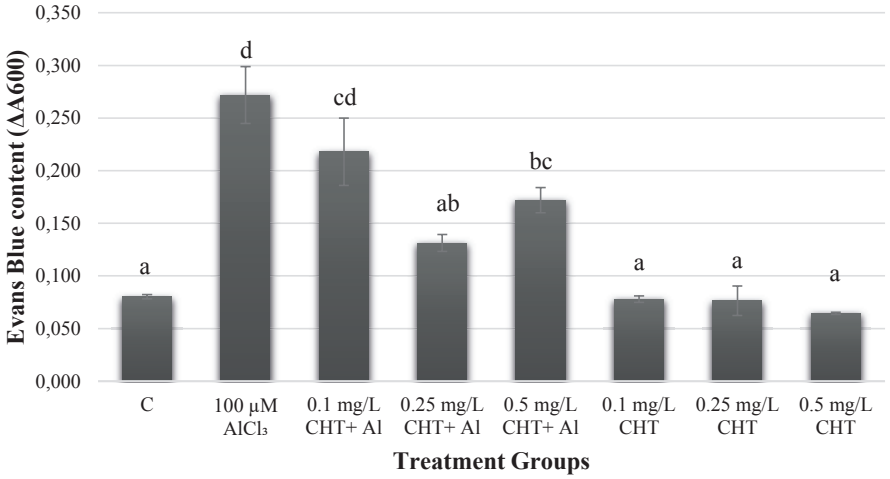


Figure 5. Loss of plasma membrane integrity of control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

Under stress conditions, lipid membranes are subjected to lipid peroxidation and are usually measured with an end product MDA. MDA content was increased by 2.94 fold in Al to control. It was decreased by 12% in 0.1 mg/L CHT + Al, 18% in 0.25 mg/L CHT + Al and 46% in 0.5 mg/L CHT + Al as compared to Al application. Besides, it was increased by 1% in 0.1 mg/L CHT, 16% in 0.25 mg/L and 40% in 0.5 mg/L CHT. According to our results, it was observed that CHT application decreased lipid peroxidation activity depending on the increasing concentrations (Figure 6).

Results of the mean guaiacol peroxidase activity (GPX), considered to be one of the oxidative stress factors revealed that the activity increased significantly in comparison to control by 2.42 fold in Al. It was also increased by 3.97% in 0.1 mg/L CHT + Al, 13% in 0.25 mg/L CHT + Al and 20% in 0.5 mg/L CHT + Al as compared to Al. It was also increased by 57% in 0.1 mg/L CHT, 76% in 0.25 mg/L and 2.11 fold in 0.5 mg/L CHT with respect to control. According to these results, it was observed that the CHT application increased GPX activity depending on the increasing concentrations (Figure 7).

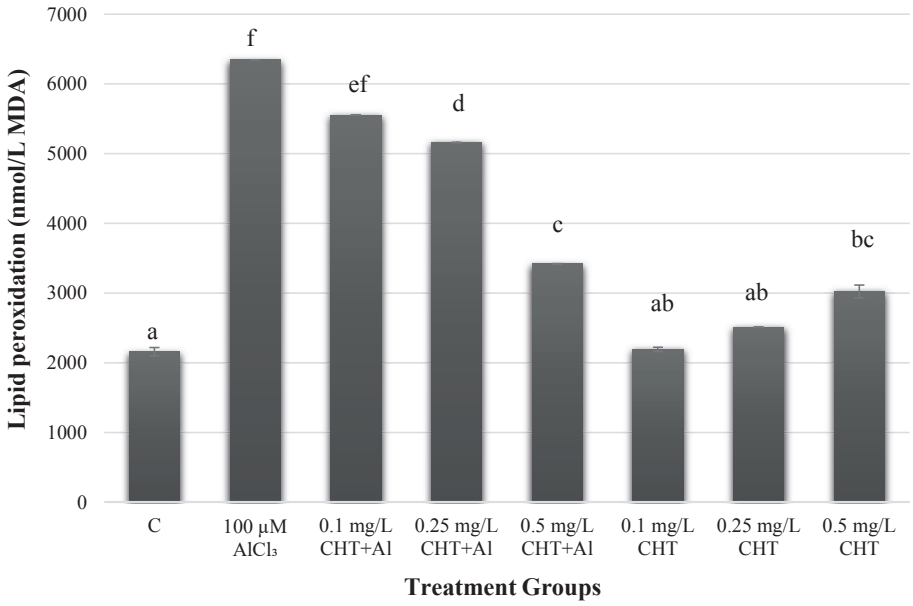


Figure 6. Lipid peroxidation (LPO) of plasma membrane integrity in control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

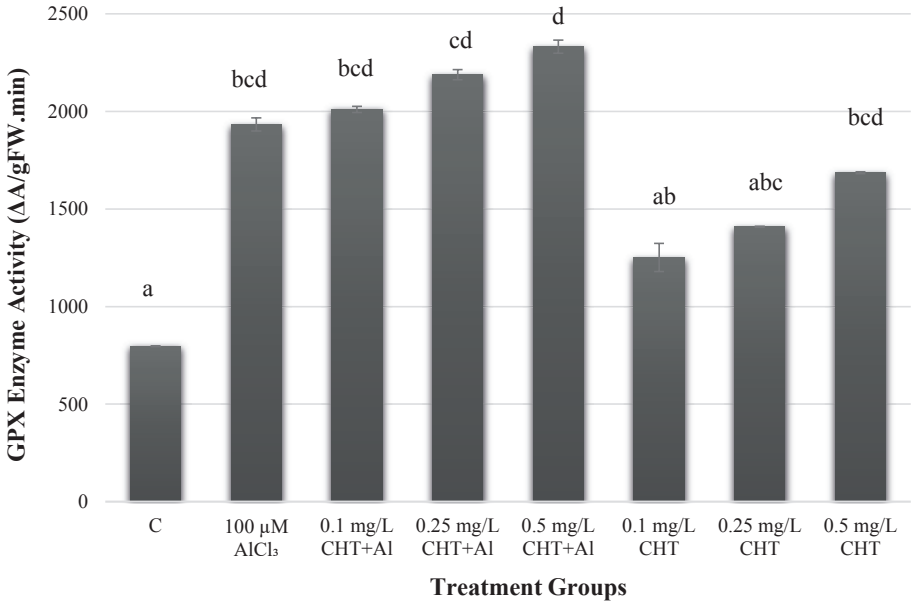


Figure 7. Guaiacol peroxidase enzyme activity in control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

One of the antioxidant enzymes, SOD is responsible for catalyzing the reduction of superoxide anions into H_2O_2. SOD activity increased by 5.18 fold in Al compared to control. It was decreased by 35% in 0.1 mg/L CHT + Al and increased by 2% in 0.25 mg/L CHT + Al and 32% in 0.5 mg/L CHT + Al as compared to Al. It was also increased 6.18 fold in 0.1 mg/L CHT, 7.17 in 0.25 mg/L and 5.51 fold in 0.5 mg/L CHT with respect to control (Figure 8).

According to total protein content (Figure 9) results it was increased by 52% in Al with respect to control. It was decreased by 33% in 0.1 mg/L CHT + Al, 12% in 0.25 mg/L CHT + Al and 3% in 0.5 mg/L CHT + Al as compared to Al. Besides it was increased by 32% in 0.1 mg/L CHT, 7% in 0.25 mg/L and 37% in 0.5 mg/L CHT with respect to control.

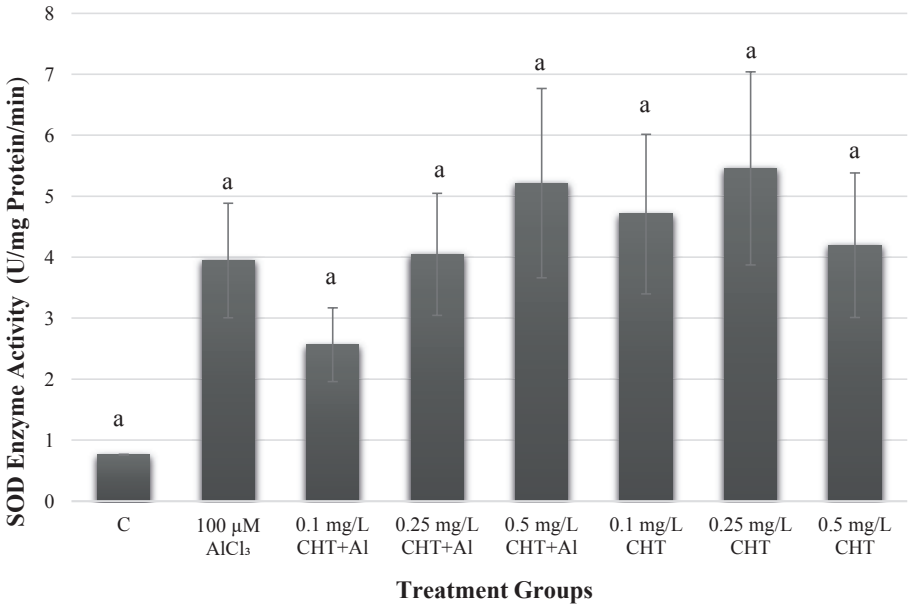


Figure 8. Superoxide dismutase enzyme activity in control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

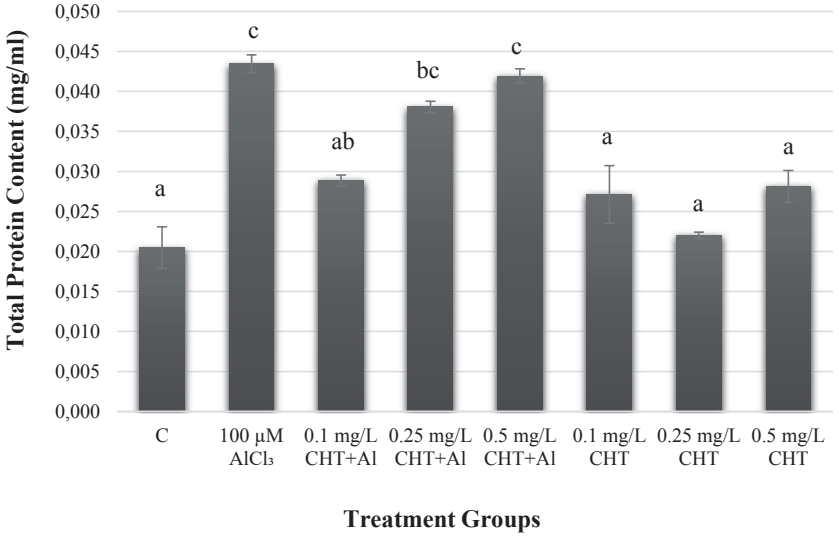


Figure 9. Total protein content in control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

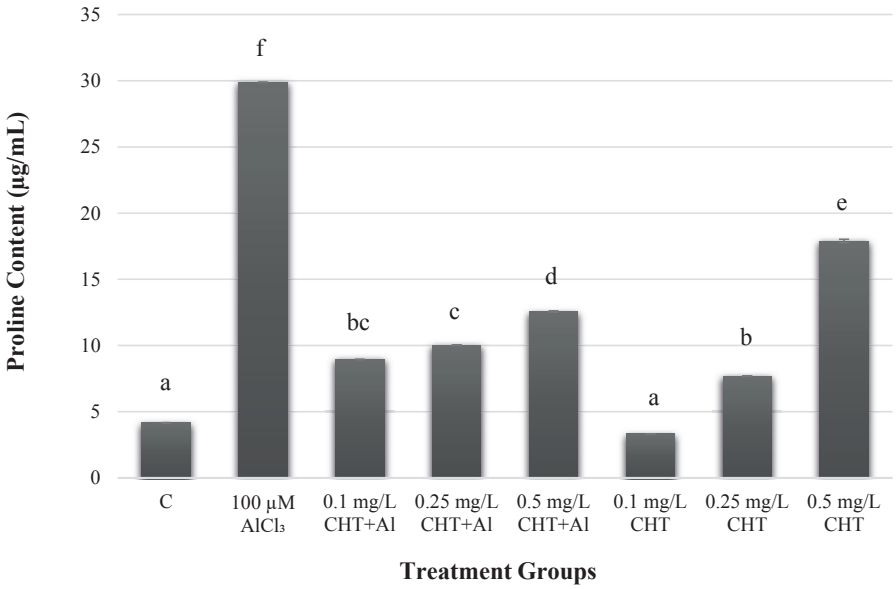


Figure 10. Proline content in control and treated wheat roots at 96 h. The data with different letter are significantly different at $P < 0.05$. The bars on columns represent means ± SD.

Proline is considered a nonenzymatic antioxidant, mitigating the adverse effects of reactive oxygen species. According to our results, the content of proline increased by 7.18 fold in Al compared to control. Although it was decreased by 70% in 0.1 mg/L CHT + Al, 66% in 0.25 mg/L CHT + Al and 57% in 0.5 mg/L CHT + Al as compared to Al, there was an increment compared to control. According to these results, the most effective group in regulating the increased amount of proline was the 0.1 mg/L CHT group. Besides it was decreased by 20% in 0.1 mg/L CHT and increased by 84% in 0.25 mg/L and 4.3 fold in 0.5 mg/L CHT with respect to control (Figure 10).

Discussion

Although aluminum (Al) is not considered as an essential element, it is one of the most abundant minerals in the soil. When the soil pH reduces below pH 5, Al complex solubilizes into phytotoxic forms (Matsumoto, 2000). Aluminum toxicity is the primary growth-limiting factor for plants in acid soils in a range of 10–100 µM (Foy 1992; Čiamporová 2002). It has been widely known that roots are the first target of Al toxicity. Thus, most toxicity studies have been built on root-Al interaction (Yanık & Vardar, 2015; Vardar et al., 2016). In this study, CHT was applied to the roots of wheat to improve Al toxicity and its effects were compared using various cellular and biochemical analyses.

Nowadays, the use of synthetic products such as fertilizers, pesticides, and growth regulators in agriculture can change the ecological balance and cause a public health problem. For this reason, natural products to be used for improvement in agriculture are very important. Since CHT is a natural biopolymer, it has been used in agriculture in recent years. CHT appears to be a promising material for cultivation under stress conditions.

The growth parameters are essential to understand the impacts of Al on plants. Our results revealed that Al inhibited root elongation progressively, but CHT application ameliorated root growth. Chibu et al. (2000) observed positive effects of CHT on the growth of several crops. Researchers have investigated the effects of CHT applications to the soil before planting, on the growth of radish, soybean, upland rice, tomato, and lettuce. According to their results, the growth of radish, soybean, and upland rice were improved more by 0.5% CHT application, but tomato and lettuce were improved more by 0.1% CHT application. Sathiyabama et al. (2016) investigated the effect of CHT on growth, yield, and curcumin content in turmeric under field conditions. Growth parameters such as shoot height, leaf number, and fresh weight were increased with the application of CHT. Similarly Stone (2020) reported that there is an improvement in growth parameters in chili pepper plants (*Capsicum annuum* L.) after the foliar application of CHT.

Lignin is the second most prevalent biopolymer after cellulose which is present in plant cell walls. It has important roles such as strengthening the cell wall, transporting minerals from the xylem, becoming a barrier against pathogens, and responding to various environmental stresses (Liu et al., 2018). Bhaskara et al. (1999) revealed that there is an increase in precursors of lignin such as p-coumaric, ferulic, and sinapic acids

by applying CHT to wheat seeds. The researchers tried to improve the seed-borne *Fusarium graminearum* Schwabe [telemorph *Gibberella zeae* (Schwein) Petch] infection by applying CHT at the rates of 2-8 mg/mL in wheat seeds. As a result, improving the infection and increasing the resistance of the seedlings was occurred possibly by stimulating phenolics and lignin accumulation. Therefore, the researchers stated that CHT has an alleviation effect on seed quality and crop yield. According to our observation, while the most accumulation was observed in Al, in CHT applied groups the accumulation increased depending on concentration. Therefore, the increase of lignin accumulation may be related to the stimulation of plant defense systems.

The formation of callose, which is one of the sensitive stress factors, was revealed with the fluorescent dye aniline blue in bright spots. As a result of Al toxicity, calloses are synthesized in the plasma membrane, mainly at the root tips (Ünal et al., 2013). Vardar et al. (2011) reported callose accumulation in *Zea mays* L. roots after application of different concentrations (150, 300 and 450 μM) of $AlCl_3$. According to our results, callose deposition was monitored in all Al treated samples. While the most accumulation was observed in Al, in alleviation groups the accumulation of callose decreased.

Hematoxylin staining is used for the localization of Al in the root tissue of the plant and is the basis of a rapid method of relative Al tolerance among wheat varieties (Ownby, 1993). Hematoxylin staining has been commonly used for direct imaging and localization of Al in root tissues. In wheat, this technique is a provider in identifying tolerant and sensitive genotypes after a very short exposure time of seedlings to Al. Hematoxylin dye turns blue-purple when it forms a complex with Al so that after staining of the root apex, the color intensity becomes a direct and quantitative measure of Al uptake. Yu et al. (2009) have observed Al accumulation in pea plants with hematoxylin staining. Researchers tried to improve the effects of Al-induced stress in pea plants by applying boron (B). When they stained the roots with hematoxylin, they obtained application B reduces Al accumulation in the roots. Similarly, Pandey et al. (2013) observed that salicylic acid alleviates Al toxicity by reducing Al uptake in rice seedlings. According to the reported studies, it has been observed that the accumulation of Al increases especially in root cells under Al stress supporting our results. Also, the researchers reported that Al accumulation in plants decreased by using various agents/chelators. In our study, applying low rates of CHT (0.1 mg/L) decreased Al accumulation more than other concentrations.

The loss of the plasma membrane integrity is a biochemical marker indicating cell death and evidence of stress symptoms. Evans blue is a substance that enters the cell and performs the staining function only after the plasma membrane structure is damaged. For this reason, it is frequently used in the analyses of disruption of cell membrane integrity. Pandey et al. (2013) observed salicylic acid alleviates Al toxicity by the restoration of root plasma membrane integrity in rice seedlings. We also observed Al-induced loss of plasma membrane integrity and the alleviation effect of CHT. Based on our results, 0.25 mg/L CHT was found to be more effective in reducing the loss of plasma membrane integrity.

Peroxidation of unsaturated lipids in the cell membrane is the most significant symptom of oxidative stress in animals and plants (Yamamoto et al., 2001). When multiple fatty acids are peroxidated in the membrane, malondialdehyde (MDA) is produced as an end product. MDA concentration, which can damage the structure of plant cells, is evaluated in determining lipid peroxidation. Guan et al. (2009) investigated the physiological changes occurring under low-temperature stress (15 °C) in the pre-germinated corn plant with different concentrations of CHT (0.25%, 0.50% and 0.75% (w/v)). According to their results, CHT caused a decrease in MDA content, changed the relative permeability of the plasma membrane and increased proline, POD, and CAT activities. Similarly, Yang et al. (2009) stated that the application of CHT under drought stress in apple seeds caused a decrease in MDA content. According to our results, MDA contents were higher in Al applied samples, while the CHT application decreased MDA contents.

Plants produce reactive oxygen species (ROS) by activating oxidases and peroxidases in response to some environmental changes (Bolwell et al., 1998; Schopfer et al., 2001; Bolwell et al., 2002). ROS are produced, especially in conditions such as drought, salt stress, freezing, high temperature, mechanical stress, heavy metal, and UV radiation. Highly synthesized ROS causes lipid peroxidation, oxidation of protein compounds, damage to nucleic acids such as DNA-RNA, inhibition of enzymatic reactions, and programmed cell death (Smirnoff, 1993; Sgherri et al., 1996; Büyük et al., 2012). Detoxification of free radicals can be accomplished with antioxidant enzymes such as SOD, CAT, APX, GPX, ascorbate glutathione, and glutathione reductase. Therefore, antioxidant enzyme activity analysis is very important in stress studies. Ma et al. (2012) observed the healing effect of using oligochitosan as exogenous to wheat seeds under salt stress. Plant growth was significantly inhibited while under 150 mM salt stress. According to the results obtained, a significant increase was observed in the activities of SOD, CAT, and GPX antioxidant enzymes in the leaves and a decrease in MDA with oligochitosan application. Besides, the accumulation of proline has significantly accelerated. Zong et al. (2017) examined the effects of CHT application on the photosynthesis and antioxidative defense system in turnip plants (*Brassica rapa* L.) under Cd stress. According to their results, Cd stress significantly reduced plant growth and the amount of chlorophyll in the leaves while causing an increase in MDA content in the leaves. While the plant growth is accelerated with the application of CHT from the

leaves, the photosynthesis content in the leaves is increased. Also, the MDA content of leaves under Cd stress has been reduced. The total activity of antioxidant enzymes in turnip leaves with Cd was significantly increased compared to the control group without Cd. It has been revealed that 1 kDa CHT application increased SOD by 18.8%, CAT by 25.5%, and POD by 47% compared to Cd treated plants. As a result of the literature studies, it was observed that Al caused an increase in GPX amount supporting our results. Also, it was observed that the CHT application caused an increase in GPX activity when the plant was under stress conditions.

Li & Yu (2001) investigated the effects of CHT application against brown rot caused by *Monilinia fructicola* (G. Winter) Honey in peach, senescence physiology, and quality characteristics in peach. Peaches treated with CHT (5 and 10 mg/mL) showed lower respiratory rate, less ethylene and MDA production, higher SOD activity, and better membrane integrity compared to the control group. Lei et al. (2011) observed the effect of CHT on the biosynthesis of artemisinin with sweet wormwood (*Artemisia annua* L.). After 0, 2, 4, 8, 12 and 16 hours 100 mg/L CHT application, 6 plants were selected from the application group and 6 plants were selected from the control group. While the SOD activity increased slowly in CHT treated plants, it reached the maximum amount after 16 hours supporting our results. Our results also confirmed increased SOD activity both in CHT and Al-CHT applications. Farouk et al. (2011) investigated the protective effect of humic acid and CHT in radish plants under Cd stress. According to the results, Cd stress (0, 100 and 150 mg/kg) applied to the soil a significant decrease was observed in the total amino acid amount and the amount of soluble protein in the shoots of the radish plant. However humic acid and CHT applications increased both the amount of total amino acids and the amount of soluble proteins. The most effective CHT alleviation was observed at the concentration of 200 mg/kg. As a result, symptoms of toxicity after 96 hours of 100 μM Al application to wheat roots were regression in root development, accumulation of callose and lignin, accumulation of Al ions, damage of cell membrane integrity, increase in lipid peroxidation, increase in GPX and SOD activities, increase in total protein and proline amount. Mahdavi et al. (2011) reported that CHT application improved osmotic potential tolerance with their study in the safflower plant. Proline activity increased when the osmotic potential increased to -0.8 MPa. When the water deficiency stress increased, low concentrations of CHT (0.05% - 0.4%) reduced the proline content. Researchers also stated that CHT is an effective regulator to increase seedling growth under drought conditions and plant tolerance under oxidative stress conditions.

Although CHT is used as a protective material against various plant stress factors, there is no research on alleviating Al toxicity. In the present research, the CHT application under Al stress has been performed for the first time.

In conclusion, CHT alleviates Al-induced stress symptoms especially in lower doses (0.1 mg/L and 0.25 mg/L). The results suggest that CHT can be used as an eco-friendly compound to induce defense responses against Al-induced stress of wheat. Our study is expected to contribute to future studies on the mechanism of Al toxicity and tolerance.

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