Role of chitosan on the growth, physiological parameters and enzymatic activity of milk thistle (Silybum marianum (L.) Gaertn.) in a pot experiment

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- H₂O₂
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- Soluble sugars

ABSTRACT

Salt stress is the destructive factor in plant growth and physiological activities. Using biobased stimulants, such as chitosan, is important to reduce the adverse effects of salinity. This study was carried out in a greenhouse located at the University of Tehran, in 2016. The goal of this study was to evaluate the effect of chitosan application on modification of adverse effects of soil salinity on growth and physiological characteristics of milk thistle (Silybum marianum (L.) Gaertn.). A pot experiment with a factorial arrangement of treatments was conducted based on a randomized complete block design (RCBD) with three replications Four irrigation water salinity levels were control (tap water 0.8), 4, 8 and 12 dS/m and four levels of chitosan were mixed with dry soil to yield 0, 0.01, 0.05 and 0.1% chitosan (DW/DW). The results showed that salinity reduced root dry weight; shoot dry weight, total plant biomass, and increased soluble sugars, proline content, CAT spell out first use enzyme activity and H₂O₂ concentration in leaves. The use of chitosan led to a reduction of salinity adverse effects and increased plant growth and improved physiological traits. Chitosan application at 0.01% increased chlorophyll a and total chlorophyll and at 0.05% level increased chlorophyll b compared to other chitosan treatments. The highest concentration of soluble sugars and proline was achieved by chitosan application across all salinity levels. Chitosan application under 0.01 and 0.05% enhanced the enzymatic activity and decreased H₂O₂ concentration in leaves. The results illustrated that chitosan could protect plants from salt stress damage by modulating intracellular ion concentration and by enhancing the capacity of antioxidant enzyme activities. It seems that the average concentration of chitosan as a bio-stimulant (0.01 and 0.05%) plays a positive role in reducing salinity and enhancing growth in milk thistle.

1. Introduction

Milk thistle (Silybum marianum (L.) Gaertn.) is a recognized medicinal plant belonging to the Asteraceae family and originated in the Mediterranean Basin. Silymarin, a derivative of milk thistle, has been used as an herbal remedy to treat liver disorders for more than 2000 years (Ashraf et al., 2014). Milk thistle is commercially grown in Europe where Poland is the biggest producer of milk thistle seeds and its derivatives in the world with cultivation area of more than 2000 ha (Andrzejewska et al., 2011). In addition to silymarin, milk thistle seeds contain relatively high amounts of oil which need to be removed from the seeds before extraction of silymarin (Hadolin et al., 2001). Milk thistle is known as a low-input annual crop which is tolerant to various environmental stresses (Karkanis et al., 2011; Afshar et al., 2015).

Salinity is one of the major abiotic stresses limiting crop production, particularly in arid and semi-arid regions. Salt stress affects plant physiology at both whole plant and cellular levels through osmotic and ionic stress (Murphy and Durako, 2003). Salinity also reduces the plant growth and development through specific ion effects, nutritional imbalance, low osmotic potential of soil solution, and combinations of these factors (Ashraf and Harris, 2004). All of these factors caused by high salt contents can affect various major plant processes like photosynthesis, protein synthesis and also energy and lipid metabolisms (Li et al., 2008). Ghavami and Ramin (2008) showed that growth...
parameters such as plant height, the number of leaves per plant, the number of capitula per plant, main shoot capitulum diameter, and seed yield and yield components per plant were reduced with salinity greater than 9 dS/m. In another study, they reported that percentage of germination and the number of normal seedlings at different salt treatment at 15.8 °C were higher than at 25 °C or 35.8 °C. The mean time to 50% germination was lowest at 15.8 °C temperature. Their results suggested that best germination indices and seedling emergence (50%) were achieved at salinity levels up to 9 dS/m at 15.8 °C (Ghavami and Ramin, 2007). Root length of milk thistle decreased as the level of water salinity increased compared to control. A negative correlation between germination and salinity level was reported by El-Garhy et al. (2016).

Application of biostimulants is one of the approaches to decrease the negative effect of abiotic stress and increase yield and quality of many crops. Chitosan can be obtained by partial deacetylation of chitin (poly-N-acetyll-β-glucosamine) from crustacean shells and it is the second most abundant polysaccharide after cellulose. There are an estimated 10 gigatons of chitin recycled in nature each year (Ruiz-Herrera et al., 2002). Chitosan is a biodegradable, renewable polysaccharide that generally is considered to be biocompatible and non-toxic (Kean and Thanou, 2010).

Chitosan was first categorized as an elicitor in plants activating genes that underlie the biosynthetic pathways of secondary metabolites (Yin et al., 2011). Chitosan can be used both in vivo and in vitro and can be sprayed on plant aerial organs to induce the accumulation of bioactive secondary metabolites (Yin et al., 2011). The report indicated that chitosan reduced plant transpiration in pepper plants, resulting in 26%–43% reduction in water use without a reduction in dry matter yield (Bittelli et al., 2001). These results suggested that chitosan might be an effective antitranspirant for reducing the consumption of irrigation water in agriculture.

Reactive Oxygen Species (ROS) contain superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide that are generated as by-products of metabolic processes inside cells or in environmental sources. Antioxidant activity of chitosan has also been described (Park et al., 2004). Chitosan modulates the plant response to several abiotic stresses including salt and water stress (Dzung et al., 2011; Ruan and Xue, 2002). Feng et al. (2007) indicated that water-soluble chitosan is a natural antioxidant and that its antioxidant activity depends on its molecular weight.

The effectiveness of chitosan application depends on the concentration of the compound, substrate, water content, temperature, stage of rooting and plant development during exposure to drought stress. Chitosan application improved the chlorophyll content, number of nodes and root establishment in grapevine plant under drought stress (Gornik et al., 2008). The research carried out by Borkowski et al. (2006) indicated that chitosan increased fruit yield of tomato and cabbage. Chitosan affects biochemical reactions which modify the adverse effects of salinity in plants (Amiri et al., 2016). Increased root and shoot dry weight, germination, leaf area index and chlorophyll content in maize and bean crops have been affected by chitosan (Amiri et al., 2016). Photosynthetic pigments and leaf relative water content are affected by salinity. Salt reduces the amount of chlorophyll in chamomile (Kovacik et al., 2009; Sarani et al., 2013). The use of biological stimulators, such as chitosan, could alleviate the adverse effects of biotic and abiotic stresses.

However, few positive results of chitosan application on plant growth and development under stressed conditions have been reported. This experiment was performed due to a lack of information on the application of chitosan on milk thistle, especially in salt stress conditions. The objective of this study was to evaluate the effect of chitosan application on growth and physiological characteristics (biochemical, physiological, and enzymatic activities) of milk thistle under salinity stress.

2. Material and methods

2.1. Experimental design

A pot experiment with a factorial arrangement of treatments was conducted based on a randomized complete block design (RCB) with three replications. All pots were seeded on Oct. 10, 2016. The experimental period continued to Dec. 20, 2016. During the experimental period, pots were kept in a glass greenhouse under natural light. The minimum and maximum temperatures of the greenhouse ranged between 20 and 25 °C, respectively, and the relative humidity was kept at ~50%. Four irrigation water salinity levels were control (tap water 0.8), 4, 8 and 12 dS/m and four levels of chitosan were mixed with dry soil to yield 0, 0.01, 0.05 and 0.1% chitosan (DW/DW). Irrigation water treatments were applied all through the growing period (planting of seeds to plant physiological maturity). Water salinity levels were created by NaCl application to tap water. To maintain a constant soil EC at the four saline levels, sufficient irrigation was applied to cause leaching. Constant soil EC during the study that was produced by the four saline irrigation treatments was confirmed by measuring soil EC following irrigation of additional pots treated same four saline irrigation levels.

2.2. Chitosan characterization

Chitosan treatments were created by application of different amounts of the material based on soil dry weight in pots. To separate the rhizosphere soil from the bulk soil, a cylindrical rhizobag (13 cm diameter, 11 cm height, and 60 mesh (0.25 mm) pores), was utilized in each pot (Fig. 2a, b). The rhizobag methodology was adopted from Wenzel et al. (2001). Each rhizobag was filled with 1000 g sieved soil and fixed vertically into the pots. Rhizobags did not prevent nutrient absorption by the roots (Ohta et al., 2004). Four levels of chitosan (0% (control), 0.01%, 0.05% and 0.1%) based on the soil dry weight in the Rhizobag (1 kg/pot) were added and mixed with the soil. Chitosan with CAS Number 9012-76-4 and MDL number MFCD00161512 was purchased from Sigma-Aldrich, USA. The physicochemical properties of chitosan were beige to orange color, powder form, viscosity 800–2000 cps and high molecular weight. The scanning electron microscopy (SEM) examination (a) as well as the chemical formula of chitosan (b) are presented in Fig. 1.

2.3. Soil characterization

A combined soil sample was collected from Research Station of College of Agriculture, University of Tehran, Karaj, Iran, and sieved to ≤2 mm. The sampled field was mainly allocated to cereal crops production. Soil moisture percentage, field capacity (F.C.) and permanent wilting point (P.W.P.) were determined based on the Klute (1986) method. The physical/chemical properties of the experimental soil are presented in Table 1.

2.4. Plantation

Milk thistle (Silybum marianum (L.) Gaertn.) seeds were purchased from Pak暗 Baz Seed Company, Isfahan, Iran. Fifteen seeds of milk thistle were sown 2 cm deep in each plastic pots (23 cm diameter × 24 cm height) containing 8.0 kg of soil. Following seeding, tap (0.8 dS/m) and prepared saline water (4, 8 and 12 dS/m) were added to each pot according to the corresponding treatments to reach saturation and drain to F.C.

During the growing period, to prevent excessive salt accumulation in rhizosphere, pots were irrigated with distilled water in two occasions. To prevent any drought stress incidence in fresh and saline water treatments, the soil moisture in pots were kept at 80% of the F.C. all through the experimental period.

During the experimental period, all the pots were kept inside a glass
Greenhouse under natural light. The minimum and maximum temperatures of the greenhouse were maintained at 20 and 25 °C in day and night, respectively; the RH was maintained at ∼50%. After germination, when plants reached 4-leaf stage, the seedlings were thinned to five plants per pot and in couple of weeks they were thinned to three plants per pot.

2.5. Studied traits

2.5.1. Root and shoot growth

After 72 days of planting, plants with a uniform growth were selected to continue the experiment. The plant root and shoot were cut at the base and weighed to determine the dry root and shoot weight. Samples were dried at 60 °C for 48 h, and their mean root and shoot dry weight were recorded for each treatment and replicate.

2.5.2. Leaf chlorophyll content

Leaf chlorophyll was measured 72 days after planting. Total chlorophyll, as well as chlorophyll a and b concentrations, were measured according to Arnon (1986). One gram of fresh leaves was taken in the middle of the flowering period, ground with 10 mL of 80% acetone and then centrifuged at 5000 rpm for 5 min. The absorbance of the solution was read at 645 nm and 663 nm against the solvent (acetone) blank using a UV-160 A UV–vis recording spectrometer (Shimadzu UV 180). The total chlorophyll and its components were calculated using the following equations.

\[
\begin{align*}
\text{Chlorophyll a} & : 12.7 (A663) - 2.69 (A645) \\
\text{Chlorophyll b} & : 22.9 (A645) - 4.68 (A663) \\
\text{Total Chlorophyll} & : 20.2 (A645) + 8.02 (A663)
\end{align*}
\]

2.5.3. Determination of soluble sugar

After 72 days of saline water (NaCl) application, soluble sugar was measured by the following procedure: 0.5 g of leaf samples were cut up and heated at 100 °C for 30 min in 5 mL distilled water. The extract was diluted 5-fold for determination. A mixture of 500 μL diluents, 1 mL 5% phenol and 5 mL sulfuric acid was made and after standing for 3 min, the absorbance was read at 485 nm. Soluble sugar concentration was quantified by comparison with a standard curve using the criterion of glucose.

2.5.4. Proline content

Proline was extracted according to the procedure of Irigoyen et al. (1992) using 0.3 g of leaf sample and 6 mL of extraction medium. Proline was quantified by spectrophotometry at 515 nm by a

Fig. 1. (a) SEM image of HM (high molecular) Chitosan and (b) Chemical formula of chitosan.

Fig. 2. (a) Rhizobag structure, (b) Vertically set into pot.
colorimetric reaction with ninhydrin (Irigoyen et al., 1992). The reaction mixture contained 1.5 mL of 25% (w:v) ninhydrin, 1.5 mL acetic acid and 0.5 mL of the extract. Samples were incubated for 1 h in a boiling water bath, and thereafter they were cooled on ice. Then 2 mL toluene were added to the reaction mixture, vigorously agitated and finally the upper organic phase was extracted to measure the absorbance. For the calculation of proline concentration, a standard curve was prepared with L-proline.

2.5.5 Enzyme activity

The antioxidant enzyme activities were measured using enzyme samples. Approximately 250 mg of fresh leaf tissue was randomly taken from plants in each treatment, frozen in liquid nitrogen, and stored at 80 °C for further analyses. The extraction of CAT, POD (Spell out for first use of enzymes) was acquired according to the method proposed by Klein, 1990). The activity was expressed in terms of mmol H2O2 reduced min−1 protein−1.

2.5.5.1 Catalase activity assay. CAT activity was measured by monitoring the H2O2 decomposition at 240 nm in 3 mL reaction mixture containing 50 mmol L−1 phosphate buffer (pH 7.0), 15 mmol L−1 H2O2, 100 mL enzyme extract and 0.1% (V/V) Triton X-100 (Aebi, 1984). The activity was expressed in terms of mmol H2O2 reduced min−1.

2.5.5.2 Peroxidase activity assay. For characterizing the peroxidase (EC 1.11.1.7) activity, a mixture of 25 mmol L−1 phosphate buffer (pH 7.0), 0.05% Guaiacol, 10 mmol L−1 H2O2 and enzyme were prepared. This activity was determined by measuring the increase in the absorbance at 470 nm due to Guaiacol oxidation (E = 26.6 mM−1 cm−1) (Hemeda and Klein, 1990).

2.5.5.3 Quantification of H2O2. According to the method proposed by Loreto and Velikova (2001), one g fresh leaf tissue was thoroughly homogenized in liquid nitrogen and extracted with 3 mL of 1% (w/v) trichloroacetic acid (TCA). Then, it was centrifuged at 10,000 × g for 15 min at 4 °C; the supernatant was collected at the end. To the assay mixture, which contained 10 mM potassium phosphate buffer (pH 7) and 1 M potassium iodide (KI), an appropriate volume of the supernatant was added to maintain an equal amount of protein; this was followed by incubation in dark for 15 min. The absorbance was measured at 390 nm, and the content of H2O2 was determined using the standard curve; the H2O2 concentration was expressed as mg g−1 of the fresh weight (FW).

Probably other places for the consistent use of unit abbreviations (min; d; diam; normally P for probability; F.C.; etc.). Also, use of Latin name when first reference of a plant type.

2.6 Statistical analysis

The data were statistically evaluated by a two way ANOVA with two factors and their levels. Factor A: chitosan with four levels (0.1, 0.05, 0.01 and 0% (control)). Factor B: Conductivity (salinity) with four levels at p ≤ 0.05. All graphs were created using Excel.

Table 1

<table>
<thead>
<tr>
<th>PWP (%)</th>
<th>Field capacity (%)</th>
<th>Total nitrogen (mg kg−1)</th>
<th>Available phosphorous (mg kg−1)</th>
<th>Available potassium (mg kg−1)</th>
<th>pH</th>
<th>Electrical conductivity (EC) ds/m</th>
<th>Organic matter (%)</th>
<th>Soil Texture (Loam)</th>
<th>Clay (%)</th>
<th>Silt (%)</th>
<th>Sand (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.42</td>
<td>22.40</td>
<td>0.09</td>
<td>14.1</td>
<td>151</td>
<td>8</td>
<td>1.70</td>
<td>0.84</td>
<td>22.5</td>
<td>43.34</td>
<td>34.16</td>
<td></td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1 Plant height

Plant height was significantly inhibited under salinity treatments compared to control (P < 0.05) (Table 2, Fig. 3). However, plant height was increased when treated with chitosan under salt stress. At control level (no salinity stress), no significant effect of different chitosan treatments was observed. The highest plant height at 4 ds/m salinity treatment was obtained by 0.05% of chitosan application. As the severity salinity treatments increased to 8 ds/m, the highest plant height was observed at 0.05 and 0.1% chitosan application. Additionally, the highest plant height at 12 ds/m salinity level was observed at 0.01% chitosan application. Nguyen Van et al. (2013) reported a positive effect of chitosan application on plant height and leaf area in coffee plant. Also, chitosan spraying with 20–40 microgram/mL had a positive effect on total fresh weight, leaf area, plant height, root length, soluble protein and soluble sucrose in leaves, while the content of crude fiber decreased in Chinese cabbage (Ouyang and Langlai, 2003). Results revealed that plant height and leaf number per plant, both under pot and field conditions, increased with chitosan application in okra plants (Mondal et al., 2012). Abdel-Mawgoud et al. (2010) showed that chitosan application improved plant height, the leaf number, leaf fresh and dry weights, and yield in strawberry.

3.2 Root dry weight

Saline water (12 ds/m) decreased root dry weight by 55.3% compared to control (Table 3). When exposed to salt, plants showed serious cellular damage, a great decrease in biomass, root growth, water status, and photosynthesis. However, chitosan application across all the concentrations increased root dry weight compared to control. Chitosan application enhanced the salinity tolerance of the plant and alleviated the severity salinity treatments increased root dry weight compared to control (P < 0.05) (Table 2, Fig. 3). However, plant height was increased when treated with chitosan under salt stress. At control level (no salinity stress), no significant effect of different chitosan treatments was observed. The highest plant height at 4 ds/m salinity treatment was obtained by 0.05% of chitosan application. As the severity salinity treatments increased to 8 ds/m, the highest plant height was observed at 0.05 and 0.1% chitosan application. Additionally, the highest plant height at 12 ds/m salinity level was observed at 0.01% chitosan application. Nguyen Van et al. (2013) reported a positive effect of chitosan application on plant height and leaf area in coffee plant. Also, chitosan spraying with 20–40 microgram/mL had a positive effect on total fresh weight, leaf area, plant height, root length, soluble protein and soluble sucrose in leaves, while the content of crude fiber decreased in Chinese cabbage (Ouyang and Langlai, 2003). Results revealed that plant height and leaf number per plant, both under pot and field conditions, increased with chitosan application in okra plants (Mondal et al., 2012). Abdel-Mawgoud et al. (2010) showed that chitosan application improved plant height, the leaf number, leaf fresh and dry weights, and yield in strawberry.

3.3 Shoot dry weight

The maximum shoot dry weight was achieved in 4 ds/m with 0.01% chitosan concentration treatment. However, in other salinity levels, chitosan application increased shoot dry weight of milk thistle plants (Fig. 4). At control level (no salinity stress) no significant effect of different chitosan treatments was observed. The highest shoot dry weight at 4 ds/m salinity treatment was obtained by 0.01% of chitosan application.
application. At the salinity of 8 dS/m, the highest shoot dry weight was observed at 0.05 and 0.1% chitosan application levels. This is while the lowest shoot dry weight was observed at control (no chitosan application) compared to all different levels of chitosan application at 12 dS/m.

Positive and additive effect of chitosan application were reported on maize (Guan et al., 2009), okra (Mondal et al., 2012), safflower (Amiri et al., 2016) plants. The mechanism of action of chitosan on growth is not clear. It was also found that chitosan may induce a signal to synthesize plant hormones such as gibberellins and enhance growth and development by some signaling pathway related to auxin biosynthesis (Uthairatanakij et al., 2007). Moreover, plants treated with chitosan may be less prone to stress evoked by unfavorable conditions, such as drought, salinity, low or high temperature (Jabeen and Ahmad, 2013; Pongprayoon et al., 2013). Chitosan leads to physiological and biochemical changes through the stimulation of biological processes, which ultimately leads to changes in the molecular level and the expression of the gene (Limpanavech et al., 2008; Nguyen Van et al., 2013; Salachna and Zawadzinska, 2014).

3.4. Total biomass

As we hypothesized, application of chitosan alleviated the negative impact of salt stress on total dry weight of milk thistle. Application of chitosan with 0.01% concentration resulted in 37.41% higher biomass on plants grown under 12 dS/m salt stress treatment, compared to control (Fig. 5). Furthermore, plants treated with chitosan with 0.01% concentration exhibited higher tolerance to salt stress in 4 dS/m level. At control level (no salinity stress) no significant effect of different chitosan treatments was observed. The highest total biomass at 4 dS/m salinity treatment was obtained by 0.01% of chitosan application. As the salinity treatments increased to 8 dS/m, the highest total biomass was observed at 0.05 and 0.1% chitosan application treatments. At 12 dS/m salinity level, the lowest total biomass was observed at control (no chitosan application) compared to all different levels of chitosan application.

This polymer has been reported to enhance and regulate plant growth, development, and yield (Gornik et al., 2008; Cabrera et al., 2013; Wang et al., 2015). Chitosan has been used as a bio promoter to stimulate plant growth, an abiotic water stress modifier, and a pathogen resistant agent. However, these responses are complex and depend on chitosan-based structures and concentrations as well as the plant species and developmental stage (Qavami et al., 2017). Application of chitosan to some extent modified yield reduction in plants grown under stress conditions (Emami Bistgani et al., 2017). The mechanism involved in plant growth stimulation under chitosan elicitation is not known. Chitosan application at various stages of plant development was demonstrated to stimulate plant growth and development: Seed priming with chitosan enhance seedling root and shoot growth (Manjunatha et al., 2008; Ma et al., 2014).

3.5. Effect of salt stress and chitosan on pigments

Chlorophyll content is widely used as an index of abiotic tolerance indicator in plants. Plants exposed to stressed environments such as salinity result in decreased chlorophyll concentration, thereby leading to overall growth retardation. As shown in Table 3, increasing salinity stress up to 4 dS/m did not significantly reduce chlorophyll a, b and total. With increasing of salinity stress up to 8 and 12 dS/m, the chlorophyll content in plants followed a decreasing trend. The minimum chlorophyll a, b and total chlorophyll (0.709, 0.306 and 1.015 mgg⁻¹ FW, respectively) were observed in 12 dS/m salinity level (Table 3).

Chitosan application at 0.01% concentration increased chlorophyll a (0.782 mgg⁻¹ FW) as well as total chlorophyll (1.115 mgg⁻¹ FW). Also, application of 0.05% of chitosan warranted maximum

Table 2

<table>
<thead>
<tr>
<th>Analysis of variance of growth and biochemical characteristics of milk thistle affected by salt stress and chitosan.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS</strong></td>
</tr>
<tr>
<td><strong>Block</strong></td>
</tr>
<tr>
<td><strong>Salt stress</strong></td>
</tr>
<tr>
<td><strong>Chitosan</strong></td>
</tr>
<tr>
<td><strong>Error</strong></td>
</tr>
</tbody>
</table>

*and **: Significant at 5% and 1% probability level.

**MS** = Mean square

**F value** = F ratio
chlorophyll in treated plants (0.341 mgg⁻¹ FW). These results were associated with the significant increase of magnesium and total nitrogen in the leaves (Dzung et al., 2011; Nguyen Van et al., 2013) as these are important elements in the composition of chlorophyll. The same results were also shown on other plants. Chitosan oligomer increased the chlorophyll content of soybean and peanut by 17.9% and 23.0%, respectively (Dzung and Thang, 2004; Dzung, 2005). Also, chitosan application increased chlorophyll content, photosynthetic and chloroplast enlargement in the leaves of Dendrobium orchid plants (Limpanavech et al., 2008) and coffee (Nguyen Van et al., 2013).

Chitosan appears to improve the tolerance of plants, such as safflower and sunflower (Jabeen and Ahmad, 2013) to salt stress. In this experiment, plants treated with salt, exhibited significant decreases in chlorophyll a, b and total compared with control, whereas chitosan with different concentrations led to a significant increase under NaCl stress. These results correspondence with Zou et al. (2015) observation on wheat.

3.6. Effect of salt stress and chitosan on soluble carbohydrates

Under salinity stress, soluble sugar content in leaves of milk thistle increased by 43.6% compared with control (P < 0.05) (Table 3). When the samples were treated with chitosan at different concentrations, soluble sugar content significantly increased (P < 0.05). Soluble sugar content in plants treated with 0.05% concentration of chitosan, increased by 4.0% compared to control. Zou et al. (2015) reported increasing of soluble carbohydrates by application of chitosan on wheat plants. Chitosan application induced a decline in malondialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars, proline, peroxidase, and catalase activities on Maize plants (Guan et al., 2009).

3.7. Effect of chitosan on proline

Salt stress increased proline accumulation in milk thistle leaves. The maximum proline content was observed in 12 ds/m (Table 3). Chitosan application stimulated proline accumulation. Proline levels in milk thistle leaves increased by 8.0% when treated with 0.1% chitosan compared to control. We found a synergy between imposed salt stress and chitosan application with respect to proline levels. The highest level of proline was accumulated in plants grown under 12 ds/m salt stress and sprayed with 0.1% chitosan. Thus, the effect of chitosan on modification of salt stress was due to its least stimulatory influence on proline accumulation. Application of 400 μL⁻¹ chitosan increased proline content in thyme (Thymus defenses) plants grown under drought stress (Emami Bistgani et al., 2017).

The accumulation of ions requires the accumulation of solutes in the cytosol playing a role in both osmoprotective and osmotic adjustment under abiotic stress (Flowers and Colmer, 2008; Munns and Tester, 2008). This accumulation of osmolytes especially that of proline, is a common phenomenon in plants. Besides its role as an osmolyte, proline contributes to scavenging ROS, stabilizing subcellular structures, modulating cell redox homeostasis, supplying energy and functioning as a signal (2008; Szabados and Savoure, 2010; Sharma et al., 2011). Proline is accumulated preferentially in leaves in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress (Silva-Ortega et al., 2008). Proline plays also a potential role in scavenging ROS products (Soshinkova et al., 2013).

3.8. Effect of chitosan application on enzymatic activities

The activities of several representative antioxidant enzymes, including CAT, POD concentration were measured in milk thistle to determine the physiological effect of exogenous chitosan on these antioxidant enzymes within the context of salt stress. The results showed

![Graph showing the effect of salt concentration on plant height of milk thistle.](image)

**Fig. 3.** Effect of salt stress and exogenous chitosan on plant height of milk thistle.

### Table 3
Growth and biochemical characteristics of milk thistle affected by salt stress and chitosan.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root dry weight (g)</th>
<th>Ch a (mgg⁻¹ FW)</th>
<th>Ch b (mgg⁻¹ FW)</th>
<th>Total Ch (mgg⁻¹ FW)</th>
<th>Soluble carbohydrates (mgg⁻¹ FW)</th>
<th>Prolin (mgg⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ds/m</td>
<td>0.539 a</td>
<td>0.811 a</td>
<td>0.353 a</td>
<td>1.165 a</td>
<td>61.403 c</td>
<td>1.512 c</td>
</tr>
<tr>
<td>0.277 b</td>
<td>0.810 a</td>
<td>0.350 a</td>
<td>1.160 a</td>
<td>62.550 c</td>
<td>1.585 c</td>
<td></td>
</tr>
<tr>
<td>8 ds/m</td>
<td>0.725 c</td>
<td>0.324 b</td>
<td>1.066 b</td>
<td>72.966 b</td>
<td>2.157 b</td>
<td></td>
</tr>
<tr>
<td>12 ds/m</td>
<td>0.241 c</td>
<td>0.306 c</td>
<td>1.015 c</td>
<td>88.180 a</td>
<td>2.707 a</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Control</td>
<td>0.333 b</td>
<td>0.757 c</td>
<td>0.329 b</td>
<td>1.087 b</td>
<td>69.867 c</td>
<td>1.890 b</td>
</tr>
<tr>
<td>0.05 %</td>
<td>0.369 a</td>
<td>0.782 a</td>
<td>0.333 b</td>
<td>1.115 a</td>
<td>70.831 bc</td>
<td>2.029 a</td>
</tr>
<tr>
<td>0.1 %</td>
<td>0.355 a</td>
<td>0.768 b</td>
<td>0.341 a</td>
<td>1.109 a</td>
<td>72.661 a</td>
<td>2.001 a</td>
</tr>
<tr>
<td></td>
<td>0.354 a</td>
<td>0.764 bc</td>
<td>0.331 b</td>
<td>1.095 b</td>
<td>71.745 ab</td>
<td>2.041 a</td>
</tr>
</tbody>
</table>

Different letters in each column for each factor indicate significant difference at P ≤ 0.05. by Duncan test.
salt stress and chitosan application had significant effect on CAT (P < 0.01). While interaction effect of salt stress and chitosan application had significant effect on POD (P < 0.05) (Table 4). With increasing salinity levels CAT activity was increased (Fig. 6). Maximum CAT activity (3.0315 IU mg\(^{-1}\) protein) was achieved in 12 dS/m. Similar to salt stress, chitosan application increased CAT activities in all levels of application (Fig. 7).

Chitosan application in milk thistle increased the activity of peroxidase in the leaves especially under salinity stress (4, 8, 12 dS/m) (Fig. 8). At control level (No salinity stress) no significant effect of different chitosan treatments was observed. The lowest peroxidase activity was observed at control (no chitosan application) compared to all different levels of chitosan application at 4 dS/m. As the salinity treatments increased to 8 dS/m again the lowest peroxidase activity was observed at control (no chitosan application) compared to all different levels of chitosan application. At 12 dS/m salinity treatment the highest peroxidase activity was observed at 0.01 and 0.05% chitosan application. Maximum activity of peroxidase (0.07133 IU mg\(^{-1}\) protein) was observed in 12 dS/m and 0.01% chitosan application.

3.9. Effect of chitosan application on H\(_2\)O\(_2\) concentration

Compared with the controls, salinity stress led to greater increases in H\(_2\)O\(_2\) levels in milk thistle leaves. Treatment with exogenous chitosan reduced H\(_2\)O\(_2\) salinity levels especially in 8 and 12 dS/m. However, this decrease was much smaller in control and 4 dS/m. So, there was not a significant difference between control and 4 dS/m salinity level at different concentrations of chitosan application. In 12 dS/m treatment, minimum H\(_2\)O\(_2\) concentration (1.127 mg g\(^{-1}\) FW) was achieved in 0.01% chitosan application (Fig. 9). At medium (8 dS/m) and severe (12 dS/m) salinity stress treatments the lowest H\(_2\)O\(_2\) activities were observed at 0.05% and 0.1% chitosan applications, respectively. Salt stress leads to the generation of reactive oxygen species, such as H\(_2\)O\(_2\), which cause lipid peroxidation and disturb normal cellular metabolism.

Exposure of plants to abiotic and biotic stresses is often accompanied by an increase in ROS, and consequently oxidative stress. Plants have evolved specific protective mechanisms, involving enzymatic and non-enzymatic antioxidants (Mittler, 2002). A key process in the antioxidant defense system is an enzymatic conversion of O\(_2\) free radicals into H\(_2\)O\(_2\), and it further detoxification by catalase and peroxidase to H\(_2\)O and O\(_2\) (Mittler, 2002). Our research showed that CAT and POD activities significantly increased in salt-stressed plants when treated with different concentrations of exogenous chitosan. By Enhancing the activities of CAT and POD showed better salt stress adaptation ability with exogenous chitosan application, which promoted the conversion of ROS species and reduced H\(_2\)O\(_2\) concentration in milk thistle plants. Sathiyabama et al., (2016) demonstrated chitosan sprayed plants had a higher peroxidase activity in leaves of turmeric (\textit{Curcuma longa} L.). Anusuya and Sathiyabama (2016) reported that foliar application of chitosan induced the activity levels of defense enzymes such as protease inhibitors (PI), \(\beta\)-1,3 glucanases, peroxidases (PO) and polyphenol oxidase (PPO) in the leaves and rhizomes of turmeric (\textit{Curcuma longa} L.) plants. Numerous studies reported that chitosan has a potential for scavenging system such as peroxidase; polyphenol oxidase (PPO)
superoxide dismutase and catalase (Agrawal et al., 2002; Ma et al., 2014).

Chitosan suppresses antioxidant enzyme activities for mitigating salt stress in mung bean varieties (Rani Ray et al., 2016). Results show that free radical scavenging activity of chitosan was increased via the amination process (Tamer et al., 2017). The promotion of antioxidant activity was attributed to replacing hydroxyl groups with free amine groups (Tamer et al., 2016). The destructive impact of reactive oxygen species (ROS) against living cells brings about damage and ultimately leads to cell death.

4. Conclusions

It is concluded that salt stress, especially under 8 and 12 dS/m, decreased growth characteristics, chlorophyll content and increased proline content, soluble carbohydrates enzymatic activity and H2O2 concentration in milk thistle leaves. However, chitosan application especially under 0.01 and 0.05% enhanced the plant growth and development, increased proline, soluble carbohydrates, enhanced enzymatic activity and decreased H2O2 concentration in leaves. The results illustrated that chitosan could protect plants from salt stress damage by enhancing the capacity of antioxidant enzymes activities. Hence, the present study demonstrated that chitosan can be used as an eco-friendly compound to protect milk thistle plants as well as to enhance growth and biochemical parameters under salinity condition.

Acknowledgments

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Table 4
Enzymatic activities of milk thistle affected by salt stress and chitosan.

<table>
<thead>
<tr>
<th>S.O.V.</th>
<th>D.F.</th>
<th>Catalase</th>
<th>Peroxidase</th>
<th>H2O2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MS</td>
<td>F value</td>
<td>MS</td>
</tr>
<tr>
<td>Block</td>
<td>2</td>
<td>0.00465</td>
<td>0.54</td>
<td>0.0000045</td>
</tr>
<tr>
<td>Salt stress</td>
<td>3</td>
<td>4.11316 **</td>
<td>480.03</td>
<td>0.0016603 **</td>
</tr>
<tr>
<td>Chitosan</td>
<td>3</td>
<td>0.085224 **</td>
<td>9.95</td>
<td>0.000111 **</td>
</tr>
<tr>
<td>Salt stress × Chitosan</td>
<td>9</td>
<td>0.008593</td>
<td>1.00</td>
<td>0.0000107 *</td>
</tr>
<tr>
<td>Error</td>
<td>30</td>
<td>0.008568</td>
<td>–</td>
<td>0.00000459</td>
</tr>
<tr>
<td>C.V.</td>
<td>–</td>
<td>4.08</td>
<td>–</td>
<td>4.06</td>
</tr>
</tbody>
</table>

*a and **: Significant at 5% and 1% probability levels.

Fig. 6. Effect of salt stress on catalase activity of milk thistle.

Fig. 7. Effect of exogenous application of chitosan on catalase activity of milk thistle.

Fig. 8. Effect of salt stress and chitosan application on peroxidase activity of milk thistle.
Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jmarap.2018.06.002.

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