

Chitosan nanoparticle and pyridoxine seed priming improves tolerance to salinity in milk thistle seedling [*Silybum marianum* (L.) Gaertn.]

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Abstract

Application of growth regulators plays important role under salt conditions. Perspectives to overcome these limitations by chitosan nanoparticle (CSNP: 0, 0.25, 0.5, and 1%) and pyridoxine (PN: 0, 0.03, 0.06, and 0.09%) seed priming was studied in both experiments with milk thistle seeds exposed to NaCl as salt stress (0, 50, 100, and 150 mM). Salinity threshold and EC₅₀ (the salinity level that 50% of germination reduction) achieved 74.85 and 213.5 mM, respectively. A significant reduction in germination percentage (49.12%), seedling length (50.07%), and seedling vigor index (67.39%) while, a significant increase in superoxide dismutase activity (54.63%) were achieved at 150 mM NaCl in compared to the control treatment. The highest germination rate was resulted by 100 mM NaCl and 0.25% CSNP and the least (2.86 seed/day) by 150 mM NaCl and without CSNP. The salt stress significantly decreased photosynthetic pigments; however, the largest value of chlorophyll a, b, and total was related to without NaCl and 1% CSNP and the least value of traits (6.1, 1.67, and 7.77 µg/g FW) to non-application of CSNP under 150 mM NaCl. PN application was caused decrease in free proline content compared to the non-application treatment. The most pronounced effects of CSNP and PN were recorded in 0.25 and 0.09% concentrations, respectively. The finding of this study leads to the conclusion that seed priming with CSNP and PN by improving physiological mechanisms such as photosynthetic pigment synthesis, antioxidant enzyme activities, and free proline content increased salt tolerance in milk thistle seedling.

Keywords: free proline content; photosynthetic pigments; salinity threshold; seed vigour index; sodium chloride

Abbreviations: CSNP, Chitosan nanoparticle; PN, Pyridoxine; GP, Germination percentage; GR, Germination rate; SVI, Seedling vigor index; MGT, Mean time germination; CAT, Catalase activity; SOD, Superoxide dismutase; LSD, least significant difference; Chl, Chlorophyll

Introduction

Milk thistle (*Silybum marianum* (L.) Gaertn.) medicinal plant belonging to the Asteraceae family. The plant grows natively in the Mediterranean and is well-known in other regions in the world such as Iran (Sedghi

et al., 2010). Seeds are achenes and contain silymarin by characteristics 5-8 mm long and 28-30 g 1000 seed weight (Rosinska *et al.*, 2018).

Salt stress is one of the major reasons for plant production losses, by disorder plant growth (Lauchli and Lutge, 2012). Salinity damages related to the management applies, genotypes, and sensitivity of crop species (Farooq *et al.*, 2015). Nutrient imbalance, ionic toxicity, and oxidative and/or osmotic stresses cause decreasing plant production (Imran *et al.*, 2018). Salt stress conditions adversely affected seed germination as one of the most critical stages of plant growth (Ghavami and Ramin, 2007). Demonstrated that the salinity stress decrease germination percentage (GP) and germination rate (GR) of most crops (Ghoulam and Fares, 2001).

Priming is one of the important methods for the improvement of seed germination under salinity conditions. Seed priming as a pre-sowing technique can improve radicle emergence, GR, seedling vigour index (SVI), seedling establishment, and yield by making changes in metabolic activities in the seeds of many crops (Nasiri *et al.*, 2014). The priming method by decreasing free radicals and Malondialdehyde production and increasing antioxidant activities was moderate the negative effects of stress condition (Parmoon *et al.*, 2015). Vitamin B₆ (PN) is a critical nutrient in the human regime and also derived from the plant sources. The mutants play important role in the many processes of plant such as developmental, physiological, and metabolic. Also, cofactor and antioxidant roles reported to the vitamin under stress condition in plants (Teresa, 2011). The mutants are essential to biosynthesis pathways of several hormones as a cofactor (Raschke *et al.*, 2011). CSNP (C₁₁H₁₇O₇N₂) is a polyacetate polysaccharide produced as a natural polymer and from Alkaline N-deacetylation of chitin and is known to be the second most abundant polymer on earth, which is the cell wall of some fungi, insects, and also some algae are produced (Yin *et al.*, 2011). This material can be mentioned from compounds with the properties of opiatetrium that impose plant defense mechanisms against stress and improve plant growth (Gornik *et al.*, 2008; Sheikha and AL-Malki, 2011). Researchers believe that this substance has the ability to increase antioxidant enzyme activities and acts as a neutralizing ROS (Harish *et al.*, 2007). Increasing chlorophyll content in strawberries (El-Miniawy *et al.*, 2013) and rice (Pongprayoon *et al.*, 2013), catalase activity in canola (Yin *et al.*, 2008), soluble protein content in tomato and eggplant (Sultana *et al.*, 2017) and tomato (Walker-Simmons and Ryan, 1984), soluble sugars and proline content in corn (Guan *et al.*, 2009) have been reported in different studies with CSNP application. Since salinity stress is limiting factors of seed germination, and seed priming is a favourable method for decrease negative effect of salt stress, so, this research was carried out to improve tolerance to salinity in milk thistle seedling by different concentrations of CSNP and PN.

Materials and Methods

Priming and salt stress treatments

Two factorial experiments were carried out to assess the effects of CSNP (0, 0.25, 0.5, and 1%) and PN (0, 0.03, 0.06, and 0.09%) on germination traits and physiological attributes of seedling of milk thistle under salt stress (NaCl i.e. 0, 50, 100, and 150 mM) in a completely randomized design with three replications. Test procedures for two experiments have been adapted from ISTA (International Seed Testing Association) guidelines (ISTA 2009). ISTA recommends germinating milk thistle seeds on between paper or top of paper at constant or alternating temperatures, 25 or 20/30 °C, respectively. In both experiments, the seed of milk thistle was immersed in a 5% sodium hypochlorite solution for 10 min to ensure surface sterility. Then they were soaked in distilled water (as control), CSNP and PN suspension for about 6 hours at 25 °C, after being rinsed three times with distilled water. Treated seeds were shade-dried for 12 hour. CSNP and PN were purchased from Sigma-Aldrich, USA. Then the 25 seeds were placed in a Petri dish (12 × 1.5 cm) with one piece of sterilized filter paper (Whatman No.1) and 9 mL of distilled water (for control) or NaCl solution based on various treatments was added to each Petri dish. Van't Hoff formula (1) was used prepared of salinity

solution (3.2, 6.4, and 9.6 gr NaCl per liter for 50, 100 and 150 mM, respectively) (Aghighi Shahverdi *et al.*, 2017).

Formula 1) $\psi = -MIRT$

Whereas ψ : osmotic potential according to Bar; M: molarity; I: Van 't Hoff factor = 2 for NaCl; R: constant number = 0.08206 bar; T: temperature according to Kelvin

Germinated seeds were counted on the second day was done on a daily basis and finally at the end of the testing period (10 days) was calculated GP, GR, MGT, and SVI according to the following formulas (Parmoon *et al.*, 2015; Aghighi Shahverdi *et al.*, 2017).

Germination percentage

$$GP = (N \times 100) / M$$

Germination rate

$$GR = \sum Ni / Ti$$

Seed vigor index

$$SVI = GP \times \text{Mean (SL or SDW)}$$

Whereas N = sum of germinated seeds at the end of the experiment, M = total planted seeds, Ti = number of days after germination, SL= Seedling Length; SDW: seedling dry weight

Threshold and EC50 values

The threshold values indicate salinity levels (NaCl) where seed begin to experience germination-reducing effects. For this purpose, we used SALT computer program proposed by USDA (United States Department of Agriculture) (Genuchten, 1983) and EC50 (NaCl level that 50% of germination reduction) calculated by Van-Genuchten and Hoffman (1984) formula (Aghighi Shahverdi *et al.*, 2019).

Photosynthetic pigments

According to Lichtenthaler (1987) method, 0.25 g of fresh seedling sample was extracted by using 5 mL 80% acetone. The extract was centrifuged at 11000 rpm for 10 min. By using a Spectrophotometer, the extract optical density was measured at wavelengths 470, 646.8, and 663.2 nanometres to estimate photosynthetic pigments.

Proline assay

Proline was determined according to the method described by Bates *et al.* (1973). Approximately 0.5 g of fresh seedling tissue was homogenized in 10 mL of 3% aqueous sulfosalicylic acid. Then, this aqueous solution was filtered through Whatman's paper No. 2 and finally, 2 mL of filtrated solution was mixed with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100 °C. The reaction mixture was extracted with 4 mL toluene, cooled to room temperature, and the absorbance was measured at 520 nm with a spectrometer" (Aghighi Shahverdi *et al.*, 2017).

Determination of enzyme activities

To measure the enzyme activity of the total seedling, 0.1 g of fresh seedling tissue was used. In order to extract protein, 0.2 g of fresh seedling tissue was pulverized in a mortar using liquid nitrogen and then 1 mL of buffer Tris-HCl (0.05 M, pH = 7.5) was added. Obtained mixture centrifuged for 21 min at 13,000 rpm, at 4 °C and the supernatant was used for enzyme activity measurements (Sudhakar *et al.*, 2001). Catalase activity (CAT) was determined according to the method described by Kar and Mishra (1976). The 60 μ L protein extract was added to Tris buffer (50 mM, pH=7) H₂O₂ 5 mM in the ice bath, then the absorbance curve was considered at a wavelength of 240 nm" (Aghighi Shahverdi *et al.*, 2017). Superoxide dismutase activity (SOD) was determined according to the method described by Beauchamp and Fridovich (1971). About 3 mL of reaction mixture, containing 0.1 mL of 200 mM methionine, 0.01 mL of 2.25 mM nitro blue tetrazolium (NBT), 0.1 mL of 3 mM EDTA, 1.5 mL of 100 mM potassium phosphate buffer, 1 mL distilled water and 0.05 mL of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 mL riboflavin (60 μ M) and placing

the tubes below a light source of two 15 W fluorescent lamps for 15 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Absorbance was recorded at 560 nm” (Aghighi Shahverdi *et al.*, 2017).

Statistical analysis

Distribution normality of achieved data from both experiments was done according to the Kolmogorov-Smirnov and Shapiro-Wilk test. Then the studied traits were statistically analyzed by the Statistical Analysis System software (SAS Institute, Cary, NC, USA, and Version 9.2). The differences among means were separated using LSD test (least significant difference) at 0.05 statistical probability level.

Results and Discussion

Threshold value and germination loss

Figure 1 indicated threshold and slope values for milk thistle (tolerance based on germination percentage). According to the results achieved from SALT computer models, threshold, EC_{50} values (the salinity level that 50% of germination reduction) and reduction slope were 74.85 and 213.5 mM and 0.25 respectively. In the other words, the increasing 10 mM of salinity concentration caused reduction 0.25 percentage of germination percentage.

Chitosan experiment

Results indicated that the effect of salinity was significant on all studied traits such as GP, GR, seedling length (SL), seedling dry weight (SDW), seedling length vigor index (SLVI), seedling weight vigor index (SWVI), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (total Chl), CAT, SOD, and proline content. In addition, seed priming with CSNP significantly affected germination parameters and physiological attributes, however, the activity of CAT and SOD enzymes were not affected (Table 1). The interaction between salinity stress and CSNP priming was significant on MTG, GR, SDW, Chl a, Chl b, total Chl, CAT, and proline. A significant reduction in GP (49.12%), SL (50.07%) SLVI (34.76%), and SWVI (67.39%) while, a significant increase in SOD activity (54.63%) were found at 150 mM NaCl in compared to the control treatment (Table 1). On the other hand, results indicated that CSNP priming, especially at 0.25% concentration, was caused to improve germination and physiological traits. A reduction in germination parameters such as GP and SWVI was observed in 1% concentration of CSNP (Table 1). The highest value of MGT was achieved 50 and 100 mM NaCl under 1% concentration of CSNP (4.52 and 4.51 day, respectively). The lowest value of the trait (3 days) was observed in 100 mM NaCl with 0.25% concentration of CSNP (Figure 2-A). The largest GR (5.7 seed/day) was resulted by 100 mM NaCl and 0.25% CSNP and the least (2.86 seed/day) by 150 mM NaCl and without CSNP. Also, 0 and 50 mM NaCl under non-application CSNP and 0.25% CSNP had the highest value of the trait (Figure 2-B). The highest seedling dry weight (31.8 g) was achieved 50 mM NaCl and 1% CSNP and the lowest (25.13 g) in 100 mM NaCl and 0.5% CSNP (Figure 2-C).

Results indicated that the salinity stress significantly decreased photosynthetic pigments, however, the largest values of Chl a, Chl b, and total Chl (17.95, 8.97, and 26.94 $\mu\text{g/g}$ FW) was related to without NaCl and 1% CSNP and the least value of traits (6.1, 1.67, and 7.77 $\mu\text{g/g}$ FW) to non-application of CSNP under 150 mM NaCl (Figure 3-A, B, C). Salinity stress significantly increased CAT activity while CSNP application decreased CAT activity (Figure 3-D). The highest CAT activity (2.42 U/min mg protein) related to non-application of CSNP under 150 mM NaCl and the lowest activity of the enzyme to 0 and 50 mM NaCl concentrations under all treatments of CSNP (Figure 3-D). Free proline content increased by salinity stress under all CSNP treatments. Free proline content was in the range of 347.1 (150 mM NaCl and 1% CSNP) and 89.9 $\mu\text{mol/g}$ FW (control treatment) (Figure 3-E).

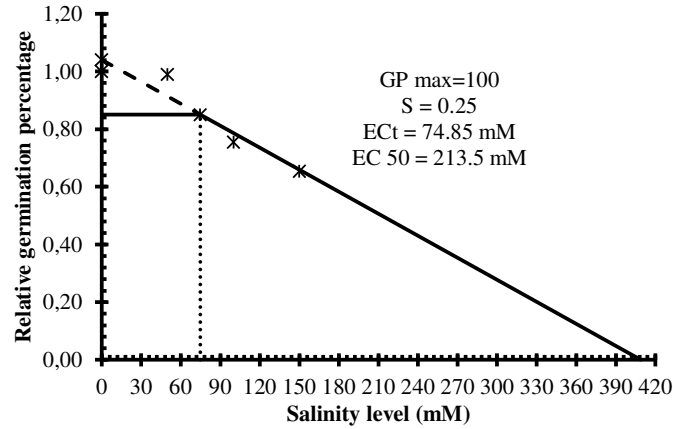


Figure 1. Threshold, slope and EC₅₀ values in milk thistle seed during germination stage

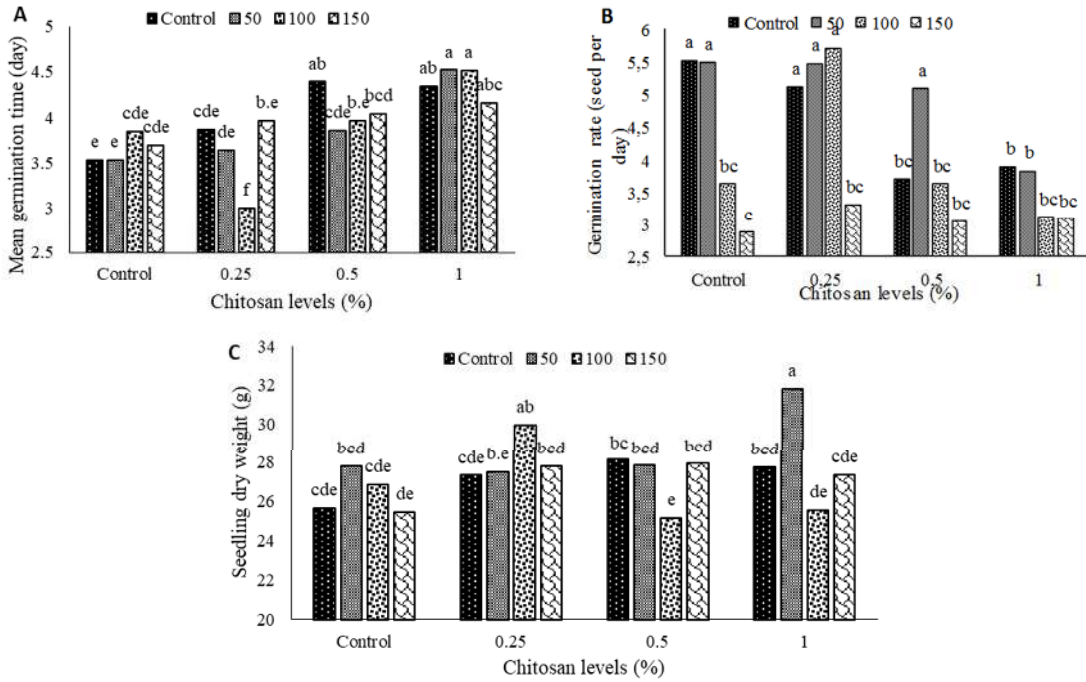


Figure 2. Effects of salinity stress (0, 50, 100, and 150 mM NaCl) and CSNP concentrations (0, 0.25, 0.5, and 1%) on mean germination time (A), germination rate (B), and seedling dry weight (C) of milk thistle seeds (means followed by the same letter are not significantly different according to LSD test at 5% level)

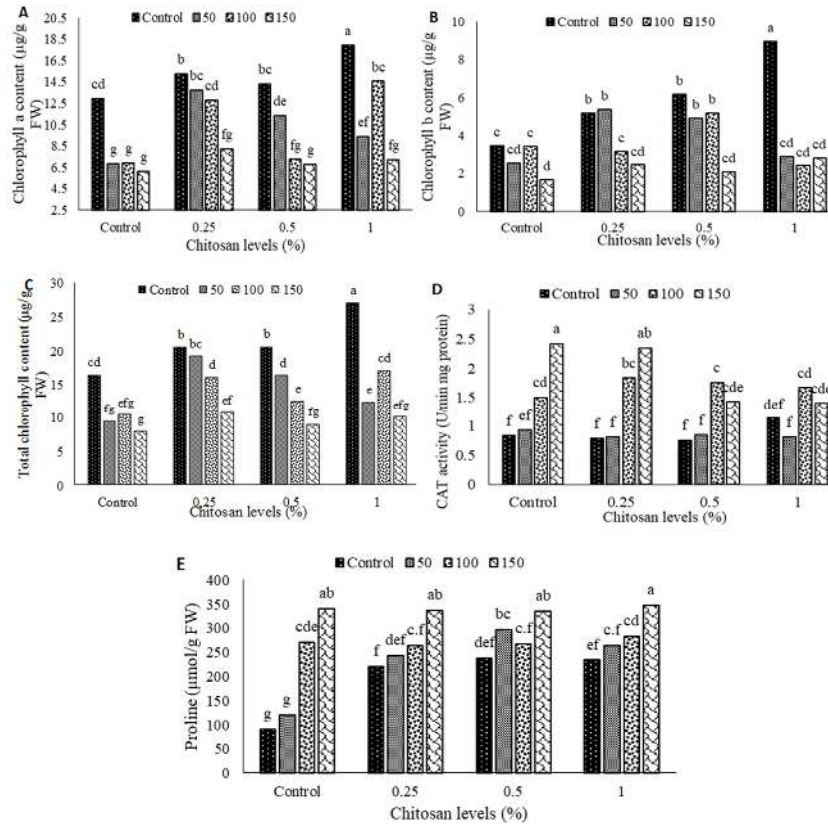


Figure 3. Effects of salinity stress (0, 50, 100, and 150 mM NaCl) and CSNP concentrations (0, 0.25, 0.5, and 1%) on chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), CAT activity (D), and proline content (E) of milk thistle seeds (means followed by the same letter are not significantly different according to LSD test at 5% level)

Table 1. Effect of different levels of salinity stress (control, 50, 100, and 150 mM) and Chitosan (control, 0.25, 0.5, and 1%) on germination indices and physiological traits of milk thistle seedling

Treatments	GP (%)	MGT (day)	GR (seed/day)	SL (cm)	SDW (g)	SLVI	SWVI	Chl a	Chl b	Total Chl	CAT (U/mg protein.min)	SOD (U/mg protein)	Proline (μmol/g FW)
								(μg/g FW)					
Salinity levels (mM NaCl)													
Control	68.0a	4.0 a	4.5 a	13.3 a	27.2b	900.9a	1844.1a	15.1a	5.9a	21.0a	0.85 b	1.9 c	195.5 d
50	69.6 a	3.89 a	4.9 a	13.1 a	28.7a	920.7a	2004.2a	10.3b	3.9b	14.2b	0.87 b	2.0 c	231.2 c
100	54.6 b	3.83 a	4.0 b	8.79 b	26.8b	491.7b	1473.6b	10.3b	3.5b	13.9b	1.7 a	2.6 b	271.5 b
150	45.6 c	3.96 a	3.0 c	6.66 c	27.1b	313.2c	1242.9c	7.0 c	2.2c	9.3 c	1.9 a	3.0 a	340.0 a
LSD (P=0.05)	5.23	0.23	0.45	2.03	1.26	162.6	166.6	1.0	0.72	1.4	0.28	0.22	24.3
Chitosan levels (%)													
Control	57.3 b	3.6 c	4.3 b	5.79 b	26.4b	350.9b	1519.3b	8.1 c	2.7b	10.9c	1.4 a	2.37 a	205.3 b
0.25	64.0 a	3.6 c	4.8 a	11.59a	28.1a	776.6a	1798.7a	12.4a	4.0a	16.5a	1.4 a	2.52 a	2.667 a
0.5	58.3 b	4.0 b	3.8 c	11.74 a	27.2ab	713.6a	1592.5 b	9.9 b	4.5b	14.5b	1.2 a	2.26 a	284.2 a
1	58.3 b	4.3 a	3.4 c	12.80 a	28.1a	785.6a	1654.1ab	12.2a	4.2b	16.5a	1.2 a	2.48 a	282.0 a
LSD (P=0.05)	5.23	0.23	0.45	2.03	1.26	162.6	166.6	1.09	0.72	1.42	0.28	0.52	24.3
Salinity × Chitosan	NS	**	**	Ns	**	NS	NS	**	**	**	**	NS	**

Means followed by the same letter in each column are not significantly different according to LSD test at 5% level

NS: non-significant; **: significant at $\alpha = 0.01$.

GP: germination percentage; MGT: mean germination time; GR: germination rate; SL: seedling length; SDW: seedling dry weight; SLVI: seedling length of vigor index; SWVI: seedling weight of vigor index; Chl a: chlorophyll a; Chl b: chlorophyll b; Total Chl: total chlorophyll; CAT: catalase activity; SOD: superoxide dismutase activity.

Pyridoxine experiment

In the PN experiment, the effect of salinity was significant on GP, MTG, GR, SDW, SLVI, SWVI, Chl a, Chl b, total Chl, CAT, SOD, and proline content. The effect of PN seed priming was significant on germination indices such as SL and SLVI and seedling physiological traits such as Chl a, Chl b, total Chl, CAT, SOD, and proline content. Interaction salinity and PN had a significant effect on MTG, SL, SLVI, CAT, and proline (Table 2). Like the CSNP experiment, 150 mM NaCl concentration was caused decreases GP (36.36%), GR (57.28%), SWVI (35.79%), Chl a (20.70%), Chl b (53.39%), and total Chl (32.58%) compared to the control treatment. On the other hand, PN application increased SWVI, Chl a, Chl b, and total Chl (Table 2).

In the interaction salinity \times PN, the highest MGT was achieved 100 and 150 mM NaCl under all PN levels. The lowest value (3.27 day) of traits related to control treatment (Figure 3-A). Results indicated that the application of PN (0.06 and 0.09% concentrations) was caused increased SL under high level of salinity. The highest SL was related to 0.09% PN priming treatment under 100 and 150 mM NaCl (15.69 and 16.1 cm, respectively) and the lowest to 0 and 0.03% PN under 100 and 150 mM NaCl (Figure 4-B). Seedling length vigor index was in the range of 988.8 (non-stressed and 0.03% PN) and 185.7 (non-application PN and 150 mM NaCl) (Figure 4-C).

The results showed that, like the CSNP experiment, an increase in CAT activity and proline content increased with salt stress. However, the highest CAT activity (0.93 and 0.94 U/min mg protein) was observed control and 0.09% concentrations of PN under 150 mM salinity treatment. The lowest value of the trait was related to control treatment (non-stressed and non-application) (Figure 5-A). PN application was caused a decrease in free proline content compared to the non-application treatment. The largest proline content (393.3 μ mol/g FW) was related to non-application of PN under 150 mM NaCl and the lowest to control treatment (non-stressed and non-application) (Figure 5-B).

Table 2. Effect of different levels of salinity stress (control, 50, 100, and 150 mM) and Pyridoxine (control, 0.03, 0.06, and 0.09%) on germination indices and physiological traits of milk thistle seedling

Treatments	GP (%)	MGT (day)	GR (seed/day)	SL (cm)	SDW (g)	SLVI	SWVI	Chl a	Chl b	Total Chl	CAT (U/mg protein.min)	SOD (U/mg protein)	Proline (μ mol/g FW)
								(ug/g FW)					
Salinity levels (mM NaCl)													
Control	66.0a	3.6 b	5.15 a	9.6 a	20.6 a	639.1a	1362.8a	19.8 a	11.5a	31.3 a	0.29 d	0.97 b	78.0 c
50	63.0a	3.7 b	4.65 a	10.4a	20.7 a	649.3a	1310.5a	20.2 a	10.9a	31.1 a	0.35 c	0.98 b	116.9 b
100	46.6b	5.1 a	2.37 b	9.5 a	20.8 a	417.2b	971.7 b	18.94a	8.26b	27.2b	0.65 b	0.96 b	132.3 b
150	42.0b	5.1 a	2.20 b	8.1 a	20.7 a	347.4b	875.0 b	15.7 b	5.36 c	21.1 c	0.89 a	1.67 a	332.7 a
LSD (P=0.05)	7.00	0.23	0.53	2.44	0.44	164.0	149.4	1.93	1.45	2.47	0.05	0.14	22.9
Pyridoxine levels (%)													
Control	55.60a	4.3 a	3.52 a	6.7 c	20.4b	357.4b	1037.2b	16.2 b	8.42b	24.7b	0.57 b	1.36 a	175.2 a
0.03	57.33a	4.4 a	3.74 a	9.3 b	20.9 a	581.2a	1201.9a	20.0 a	10.11a	30.1a	0.50 c	1.06 b	163.9ab
0.06	57.33a	4.4 a	3.68 a	9.3 b	20.6ab	537.0a	1187.6a	19.3 a	9.95 a	29.2a	0.65 a	1.07 b	144.8 b
0.09	52.33a	4.4 a	3.43 a	12.2a	20.9 a	577.5a	1093.2ab	19.0 a	7.63 b	26.69b	0.46c	1.10 b	176.1 a
LSD (P=0.05)	7.00	0.23	0.53	2.44	0.44	164.0	149.4	1.93	1.45	2.47	0.05	0.14	22.9
Salinity \times Pyridoxine	NS	**	NS	**	**	**	NS	NS	NS	NS	**	NS	**

Means followed by the same letter in each column are not significantly different according to LSD test at 5 % level
NS: non-significant; **: significant at $\alpha = 0.01$.

GP: germination percentage; MGT: mean germination time; GR: germination rate; SL: seedling length; SDW: seedling dry weight; SLVI: seedling length of vigor index; SWVI: seedling weight of vigor index; Chl a: chlorophyll a; Chl b: chlorophyll b; Total Chl: total chlorophyll; CAT: catalase activity; SOD: superoxide dismutase activity.

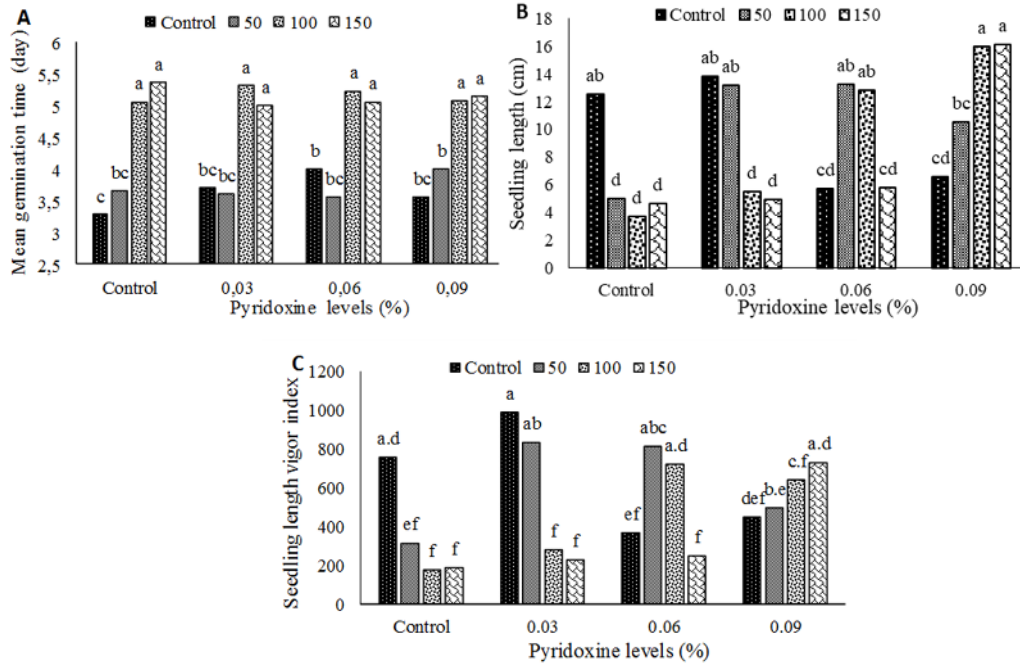


Figure 4. Effects of salinity stress (0, 50, 100, and 150 mM NaCl) and PN concentrations (0, 0.03, 0.06, and 0.09%) on mean germination time (A), seedling length (B), and seedling length vigor index (C) of milk thistle seeds (means followed by the same letter are not significantly different according to LSD test at 5% level)

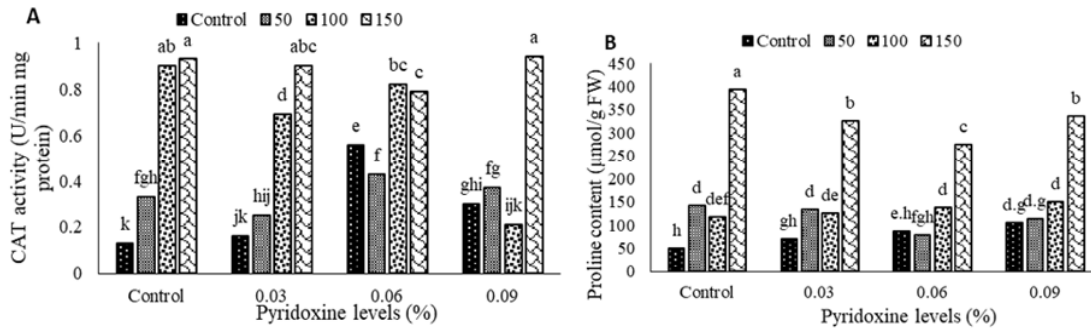


Figure 5. Effects of salinity stress (0, 50, 100, and 150 mM NaCl) and Pyridoxine concentrations (0, 0.03, 0.06, and 0.09%) on CAT activity (A) and proline content (B) of milk thistle seeds (means followed by the same letter are not significantly different according to LSD test at 5% level)

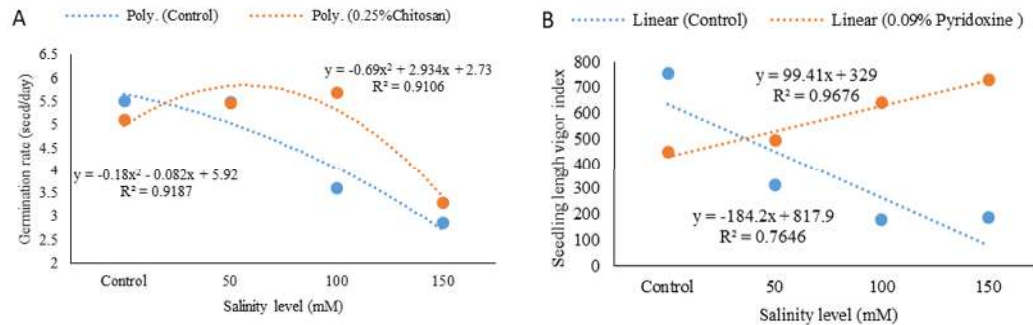


Figure 6. Germination rate (A) response of milk thistle to application of 0.25% chitosan (A) and seedling length vigor index response of milk thistle to application of 0.09% pyridoxine under different salinity levels.

Discussion

Evaluation the role of CSNP and PN seed priming on improve tolerance to the salinity stress was one of the objectives in the present investigations. In the both of experiments (CSNP and PN), salinity stress significantly decreased germination indices such as GP, GR, SL, SLVI, and SWVI and seedling physiological traits such as Chl a, Chl b, and total Chl, while the increased activity of CAT and SOD enzymes and free proline content. Enhance levels of salinity decrease the water potential and resulted water less available to the seed. Osmotic and/or ionic effects of salinity stress were caused decreasing GP and GR in compared to the non-stress condition (Ghavami and Ramin, 2007). The decrease of water content under salt condition due to declines turgor pressure in the plant, and therefore prevents cell division, cell enlargement, and plant growth (Farooq *et al.*, 2009). Based on reports of Ghavami and Ramin (2007), it may be determined that the plant at the seed germination phase may be categorized as a facultative halophyte, which confirms the results of this study.

Under salinity stress conditions, limited stomatal conductance, diminished activities of carbon fixation enzymes, reduced quantities of photosynthetic pigments, and destruction of photosynthetic apparatus are among the key factors limiting the process of photosynthesis (Qu *et al.*, 2012; Imran *et al.*, 2018). In line with our results, it has been detected a reduction in photosynthetic pigments in different plants under NaCl stress (Cha-Um and Kirdmanee, 2009; Aghighi Shahverdi *et al.*, 2017). Augmented generation of reactive oxygen species (ROS) causes damages to cellular components under salt conditions which could result in progressive oxidative damages (Sharma *et al.*, 2012; Imran *et al.*, 2018). It reported that the higher ROS increased damages to chlorophyll pigments (Verma and Mishra, 2005).

Enzymatic and non-enzymatic antioxidants response are defensive mechanisms to overcome oxidative stress (Lipiec *et al.*, 2013; Demidchik, 2015). Proline, non-enzymatic antioxidants response, is known to act as an osmolyte / osmoprotectant agent under drought or salinity stress (Lehmann *et al.*, 2010). The osmolyte has an important role in the scavenging free radicals, osmotic pressure adjustment, stabilizing sub-cellular structures and storing carbon and nitrogen (Gorzi *et al.*, 2018). The results of current study concluded that a significant increase in the free proline content and activity of CAT and SOD enzymes under salt stress especially in 150 mM NaCl level.

Seed priming is a common technique involving the direct application of plant growth regulators to seeds that are known to improve stress tolerance and increase overall seedling growth (Keshavarz and Ghol Moghadam, 2017). In the current study, the seed priming with CSNP and PN significantly enhanced some

germination indices and seedling physiological traits. In this regard, Ferreira *et al.* (2016) reported that seed priming with PN solution enhance GR and promotes more uniform germination of durum wheat in stress conditions which confirm the findings of this study. Also, it suggests that seed priming with CSNP may improve the germination rate of maize seed and benefit for seedling growth under stress conditions (Guan *et al.*, 2009).

Results demonstrated that seed priming with CSNP and PN significantly increases germination parameters and seedling physiological traits. The most pronounced effects of CSNP and PN were recorded in 0.25 and 0.09% concentrations, respectively. Seed priming by 0.25% CSNP under 100 mM NaCl increased GR up 36.6% and also, priming by 0.09% PN under 150 mM NaCl increased SLVI up 74.55% (Figure 6). Furthermore, seed priming with CSNP and PN was caused by moderating the negative effects of salinity stress. It seems that increasing photosynthetic pigments, modifying the activity of antioxidant enzymes, and increasing the synthesis of secondary metabolites, such as proline amino acid, has the effect of modulating the negative effects of salinity stress, which caused the tolerance of this plant to salt stress.

Some researchers reported that the B group vitamins are known to promote seedling growth and beneficial effect in various plant species (Keshavarz and Ghol Moghadam, 2017). Burguieres *et al.* (2007) reported that the application of vitamin B1 by increasing free proline content due to moderate the negative effect of osmotic stress. This proposes that it contributed to osmotic adjustment and cell water status adjustment, so it can play an important role in alleviating salinity stress. Moreover, this vitamin may act as a growth inducer, which can play a role in mitigating the inhibitory effects of NaCl stress on processes related to seedling growth through enhancing stimulating cell division or cell enlargement (Keshavarz and Ghol Moghadam, 2017). These results can be related to earlier studies that reported that vitamins application could promote and counteract stress-induced factors on growth inhibition in a range of crop species (Dolatabadian *et al.*, 2008). Seed priming by vitamin B12 resulted in improving plant growth and moderating effect under stress and non-stress conditions and caused a significant enhance in growth parameters in salt-stressed plants (Keshavarz and Ghol Moghadam, 2017).

Seed priming by CSNP under stress condition was caused highly improved germination parameters and reduced germination time to promote early seed germination and harmonized growth in rice (Suchada *et al.*, 2007) and maize (Guan *et al.*, 2009). Sadeghi *et al.* (2011) reported that the reserve mobilization of food material, activation, and re-synthesis of some enzymes, DNA and RNA synthesis started during osmotic priming resulted improvement in germination and vigor of soybean plant. There is a possibility that similar germination responsive genes may be activated because of CSNP priming under osmotic stress. The results of current experiment are in line with these previous reports as GP, GR, SVI, and germination energy were improved while MGT was reduced by CSNP priming (Hameed *et al.*, 2014). Anusuya and Banu (2016) stated that Chitosan significantly promoted growth and biochemical variation capabilities of chickpea. Generally, the effect of CSNP on plants is complex and many factors can affect seed germination, plant growth, and physiological parameters. In the current study, the positive effect of CSNP was observed for milk thistle seedling.

Conclusions

NaCl stress had a negative effect on seed germination and seedling growth of milk thistle. Salt tolerance threshold results showed that this seed had a favorable tolerance to salinity stress at the germination stage. Due to the decrease in the GP and GR as well as SVI occur in higher than 100 mM salinity levels. Also, seed priming with CSNP and PN has improved germination indices and seedling physiological parameters such as chlorophyll, proline, and the activity of the antioxidant enzyme. The most pronounced effects of CSNP and PN were recorded in 0.25 and 0.09% concentrations, respectively. The finding of this study leads to the

conclusion that seed priming with CSNP and PN by improving physiological mechanisms such as synthesis of photosynthetic pigment, antioxidant enzymes activity, and proline amino acid moderated the negative effects of salinity stress and increased GP, GR, and seedling growth. The co-application of CSNP and PN is recommended for further research at higher different sources of salt stress.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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