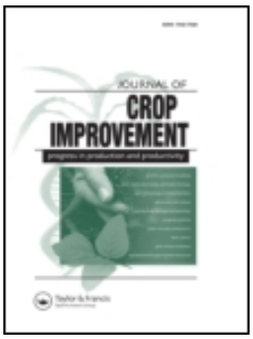

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Chitosan Improves Osmotic Potential Tolerance in Safflower (*Carthamus tinctorius* L.) Seedlings

BATOOL MAHDAVI[1],
SEYED ALI MOHAMMAD MODARRES SANAVY[1],
MAJID AGHAALIKHANI[1], MOZAFAR SHARIFI[2],
and ARIA DOLATABADIAN[1]

[1]*Agronomy Department, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran*
[2]*Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

Salinity and water-deficit stress reduce yield in agricultural crops. On the other hand, seed germination is critical in seedling establishment and subsequent plant growth. Therefore, the present investigation was carried out to evaluate the effects of chitosan (Ch) concentrations (0% [control], 0.05%, 0.1%, 0.2%, 0.4%, 0.5%, 1%, 2%, and 3%) on the tolerance of safflower (Carthamus tinctorius *L.) to different osmotic potentials (0, −0.4, −0.8, and −1.2 MPa). Induced osmotic potential significantly decreased germination percentage, germination index and rate, length and weight of root and shoot, and protein content. Proline content, malondialdehyde content (MDA), and catalase (CAT) and peroxidase (POX) activity increased when osmotic potential was increased to −0.8 MPa. Under unstressed conditions (0 MPa), there were no significant differences in germination percentage among different concentrations of chitosan, whereas MDA content, CAT, and POX activity were increased by low concentrations of chitosan (0.05%–0.4%). With increasing water-deficit stress, low concentrations of chitosan increased germination percentage but decreased MDA and proline contents and CAT and POX activity. Thus, it could be concluded that low concentrations of chitosan exhibited positive effects on water-deficit alleviation through the reduction of*

Address correspondence to Seyed Ali Mohammad Modarres Sanavy at Agronomy Department, Faculty of Agriculture, Tarbiat Modares University. Jallal-Al-Ahmad Highway, Nasr Bridge, P.O. Box 14115-336, 1411713116, Tehran, Iran. E-mail: Modaresa@modares.ac.ir

enzyme activity. Therefore, chitosan should be an effective biostimulator to enhance seedling growth and plant tolerance to oxidative stress conditions, especially under conditions of drought stress.

KEYWORDS antioxidant enzymes, chitosan, germination, osmotic potential, safflower

INTRODUCTION

Safflower, an important oil-seed plant, is one of the oldest domesticated crops. India, Ethiopia, and Iran are the leading countries with the longest tradition of safflower cultivation as an oil crop (Tuncturk & Ciftci 2004). It can be grown throughout the semiarid regions of the temperate climates in many areas of the world for several purposes, including as a vegetable, industrial oil, spice, and birdfeed (Weiss 2000; Johnston et al. 2002). Manivannan et al. (2007a) found that water deficit was an important productivity-limiting factor in various parts of the world. The lack of adequate moisture, leading to water stress, is a common occurrence in rain-fed areas, which is brought about by infrequent rains and poor irrigation arrangements (Manivannan et al. 2007b). Seed germination is usually the most critical stage in seedling establishment and determining successful crop production (Almansouri, Kinet, & Lutts 2001). The effect of unfavorable weather is probably more critical during germination and early seedling-development stages than at any other stage of vegetative growth. Successful germination of seeds under a wide range of environmental conditions (e.g., temperature and moisture) is important for early seedling establishment (El-Hendawy et al. 2005). Exposure of seeds to unfavorable environmental conditions, such as water-deficit stress, might compromise seedling establishment (Albuquerque & Carvalho 2003).

Application of biostimulators is one approach to decrease the negative effect of abiotic stress and increase yield and quality of crops (Górnik, Grzesik, & Romanowska-Duda 2008). Several substances with elicitor properties, which trigger stress responses linked to plant defense mechanisms, have been identified, e.g., chitosan (Kowalski et al. 2006). Chitosan, a most common polymer (Wojdyła 2001), is a natural nontoxic biopolymer formed by alkaline deacetylation of chitin (an important component of the shells of Crustacea, such as crab, shrimp, and crawfish) (No et al. 2002). Recently, application of chitosan in agriculture has increased (Babel & Konawa 2003) because it can affect many plant responses. Ruan and Xue (2002) showed that chitosan could accelerate seed germination and improve the tolerance of hybrid rice seedlings to stress. Seed priming with two different acidic chitosan solutions enhanced the vigor of maize seedlings (Shao et al. 2005).

Chitosan has recently attracted additional attention because of its antioxidant activities (Park, Je, & Kim 2004; Guo et al. 2005; Huang, Mendis, &

Kim 2005). Its antioxidant activity depends on the molecular weight as well as on the degree of deacetylation (Kim & Thomas 2007). Also, various studies have confirmed that chitosan might have a potential as a free radical scavenger (Kim & Thomas 2007; Yen, Yang, & Mau 2008). The chitosan antioxidant activity is described by several mechanisms (Muzzarelli, Muzzarelli, & Terbojerich 1997; Park, Je, & Kim 2004). Chitosan can scavenge OH and O_2^- radicals and has been reported to have DNA-protective properties (Harish Prashanth et al. 2007). The scavenging mechanism of chitosan may be attributed to its structure, which features large numbers of hydroxyl and amino groups available to react with reactive oxygen species (ROS) (Xie, Xu, & Liu 2001; Li et al. 2002; Sun, Xie, & Xu 2004; Feng et al. 2009). Previous studies on the antioxidant activity of chitosan have focused on its biomedical, food, and environmental protection aspects (Chung et al. 2003; Xu et al. 2007; Meng et al. 2008).

Guan et al. (2009) found that chitosan increased peroxidase and catalase activities in two maize lines. Xu et al. (2007) and Guan et al. (2009) reported that chitosan treatment decreased malondialdehyde content in *Hydrilla verticillat* and maize. Xue et al. (1998) demonstrated that chitosan could retard lipid oxidation by chelating metal ions or by combining with lipids. Thus, it seems that chitosan is a promising material for improving seed germination, especially under arid and semiarid conditions where osmotic stress limits plant growth and development. In the present study, the effects of seed priming with different concentrations of chitosan solutions were investigated. The main objective of this study was to examine the potential benefits of chitosan by reducing damage to safflower at the germination and seedling stages under water-deficit conditions.

MATERIALS AND METHODS

Safflower seeds were disinfected with sodium hypochlorite for 5 min and then 96% ethanol for 30 sec. The seeds were washed with distilled water and then soaked in various concentrations of chitosan (Ch) solutions (0% [control], 0.05%, 0.1%, 0.2%, 0.4%, 0.5%, 1%, 2%, and 3% dissolved in 1% acetic acid solution) for 3 h and then air-dried. After this, 25 seeds for each treatment were transferred to sterile glass petri dishes of uniform size lined with two layers of Whatman No. 1 filter paper treated with 10 ml of one of the PEG 6000 solutions (0, −0.4, −0.8, and −1.2 MPa). Solution osmotic potential was calculated as described in Michel (1973):

$$\text{Equation 1: Water potential (bar index)} = -(1.18 \times 10^{-2})\,C - (1.18 \times 10^{-4})\,C^2 + (2.67 \times 10^{-4})\,CT + (8.39 \times 10^{-7})\,C^2T,$$

where C is PEG concentration and T is temperature in centigrade.

To reduce the risk of infection and evaporation of solution, all the petri dishes were wrapped in parafilm. All operations were done under a laminar flow hood. Petri dishes were incubated at 20 ± 1°C under 16/8 h photoperiod. After 10 days, total number of seeds germinated and length and weight of root and shoot were measured. Germination percentage, germination index, and germination rate were calculated. Samples of seedlings were frozen in liquid nitrogen and stored at −80°C until biochemical analysis were done.

Preparation of Extracts

Frozen seedlings (0.2 g) were homogenized in a mortar and pestle with 3 ml of ice-cold extraction buffer (25 mM sodium phosphate buffer, pH 7.8). The homogenate was centrifuged at 18,000 rpm for 30 min at 4°C. Then supernatant was filtered through Whatman paper no. 4 and used for the determination of enzyme activity and protein content as crude extract.

Catalase Activity

Catalase (CAT) activity was assayed according to the method of Cakmak and Horst (1991). The reaction was initiated by adding 500 μl of 10 mM H_2O_2, 1400 μl of 25 mM sodium phosphate buffer, and 100 μl of crude enzyme extract. Decrease in absorbance was recorded at 240 nm for 1 min. Catalase activity of the extract was expressed as CAT unit: min^{-1} mg^{-1} protein.

Peroxidase Activity

Peroxidase (POX) activity was estimated by the method of Ghanati, Morita, and Yokota (2002). Peroxidase enzyme activity was determined by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance was recorded at 470 nm for 1 min with a spectrophotometer (Cintra GBC, Dandenong, Victoria, Australia). The reaction mixture contained 500 μl 28 mM guaiacol, 1900 μl 60 mM potassium phosphate buffer (pH 6.1), 500 μl 5 mm H_2O_2, and 100 μl crude extract. POX activity of the extract was expressed as POX unit: min^{-1} mg^{-1} protein.

Protein Assay

Total protein content was determined using bovine serum albumin (BSA) as a standard, according to the method described by Bradford (1976). The protein concentration was calculated from a BSA standard curve.

Proline Content

Proline content was determined following the method of Bates, Waldern, and Teave (1973). The frozen seedlings (0.2 g) were homogenized in 3% sulfosalicylic acid, and the homogenate was centrifuged at 10,000 rpm. Supernatant was used for the estimation of proline content. The reaction mixture consisted of 2 ml of acid ninhydrin and 2 ml of glacial acetic acid, which was boiled at 100°C for 1 h. After termination of the reaction in an ice bath, the reaction mixture was extracted with 4 ml of toluene; absorbance was read at 520 nm.

Lipid Peroxidation Assay

The level of membrane damage was estimated by measuring malondialdehyde (MDA) as the last product of peroxidation of membrane lipids (De Vos et al. 1991). Samples (0.2 g) were homogenized in an aqueous solution of trichloroacetic acid (10% w:v), and aliquots of the filtrates were heated in 0.25% thiobarbituric acid. The amount of MDA was determined from the absorbance at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using the extinction coefficient of MDA ($\varepsilon = 155\ \mu M^{-1}$ cm).

Statistical Analysis

Experimental treatments were arranged as factorial in a randomized complete-block design with three replications. All data were analyzed using SAS software (SAS Institute 2002). When analysis of variance (ANOVA) showed significant treatment effects, the least significant differences (LSD) test was applied to compare the means at $P < 0.05$.

RESULTS

The main effects of osmotic potentials and chitosan concentration and two-way interaction between them were significant for all studied traits, except for the effect of chitosan concentrations on POX activity. The results showed that with increasing water stress level, germination percentage, germination index and rate, and length and weight of root and shoot decreased (Table 1). Germination percentage and shoot length were not affected by different concentrations of Ch under unstressed conditions (0.0 MPa). Also, the highest and the lowest germination index and rate were observed in 0.5% and 1% Ch concentrations, respectively. The longest root length was found at 0.05% Ch treatment. The application of different Ch concentrations increased shoot and root dry weight compared with the control in the unstressed seedlings (Table 1).

TABLE 1 Effect of Different Chitosan Concentrations and Osmotic Potential on Seed Germination Parameters

Osmotic potential (MPa)	Chitosan concentration (%)	Germination percentage (%)	Germination index	Germination rate	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)
0	0 (Control)	80.00a	18.06b	41.67ab	2.92a	2.67ab	0.066d	0.010d
	0.05	81.33a	19.11ab	43.92ab	2.68a	3.21a	0.086cd	0.016ab
	0.1	82.67a	18.06b	41.69ab	2.61a	3.07ab	0.110ab	0.012cd
	0.2	81.33a	18.67ab	42.87ab	2.85a	3.10ab	0.111ab	0.015ab
	0.4	82.67a	18.25ab	42.08b	2.86a	2.78ab	0.107abc	0.016a
	0.5	85.33a	21.33a	48.71a	2.72a	2.75ab	0.120a	0.014abc
	1	80.00a	17.83b	41.33b	2.67a	2.61b	0.095bc	0.013bc
	2	89.33a	20.83ab	47.99ab	2.69a	2.68ab	0.094bc	0.015ab
	3	78.66a	18.83ab	43.24ab	2.87a	2.72ab	0.101abc	0.014abc
−0.4	0 (Control)	68.33b	13.55abc	32.78abcd	1.23a	1.77c	0.119a	0.013bc
	0.05	84.0a	14.89a	35.39ab	1.31a	2.04c	0.112ab	0.014bc
	0.1	84.66a	15.78a	37.19a	1.31a	2.60ab	0.108ab	0.015abc
	0.2	85.67a	14.0ab	32.77abcd	1.33a	3.02a	0.107ab	0.017ab
	0.4	87.33a	14.53ab	34.36abc	1.27a	2.25bc	0.110ab	0.017ab
	0.5	70.33b	11.27bcd	27.38cde	1.17a	1.93c	0.100ab	0.014bc
	1	66.67bc	11.44bcd	28.43bcde	1.19a	1.97c	0.113ab	0.019a
	2	63.33bc	10.66dc	26.36de	1.19a	1.96c	0.110ab	0.019a
	3	56.00c	9.28d	23.27e	0.853b	1.93c	0.098b	0.017c

(*Continued*)

TABLE 1 (Continued)

Osmotic potential (MPa)	Chitosan concentration (%)	Germination percentage (%)	Germination index	Germination rate	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)
−0.8	0 (Control)	69.67cd	8.97ab	22.37abc	0.767ab	1.65bcd	0.098ab	0.009ab
	0.05	82.66ab	9.08ab	22.19abc	0.847a	2.15ab	0.099a	0.008bc
	0.1	80.33abc	9.86a	24.48a	0.867a	1.92abc	0.113a	0.009a
	0.2	85.67a	9.89a	24.13ab	0.853a	1.81abc	0.102ab	0.10a
	0.4	86.33a	10.19a	25.62a	0.847a	2.31a	0.112a	0.009ab
	0.5	73.33bcd	8.00bc	20.25bcd	0.787ab	1.79abc	0.077bc	0.006cd
	1	63.33de	7.08cd	17.48de	0.447abc	1.55dc	0.067c	0.008ab
	2	56.67e	6.06d	15.00e	0.373cb	1.15d	0.046d	0.005d
	3	68.00de	7.67bc	18.95cd	0.287c	1.16d	0.043d	0.004d
−1.2	0 (Control)	57.33ab	4.56bc	10.06bcd	n.d.	0.953a	n.d.	0.004b
	0.05	68.67ab	8.86a	21.47a	0.520a	1.06a	0.050b	0.007a
	0.1	70.33ab	6.50ab	15.65ab	0.220bc	0.980a	0.033c	0.004b
	0.2	72.00ab	5.50ab	11.98bc	0.393ab	0.997a	0.048b	0.004b
	0.4	76.00a	6.08ab	13.55ab	0.480a	1.03a	0.062a	0.004b
	0.5	53.33b	4.42bc	10.00bcd	n.d.	0.520b	n.d.	0.003c
	1	21.33c	1.42c	2.73d	n.d.	0.336c	n.d.	0.001d
	2	22.67c	1.53c	2.99d	n.d.	0.303c	n.d.	0.001d
	3	25.33c	1.86c	3.96cd	n.d.	0.283c	n.d.	0.001d

Means within each column of each section followed by the same letter are not significantly different at $P < 0.05$ by LSD test.

At osmotic potential of −0.8 MPa, germination percentage was increased when seeds were treated with low concentrations of Ch (0.05%–0.4%). Germination index, germination rate, and shoot dry weight at low concentrations of Ch had no significant difference compared with the control, whereas these traits were reduced at high Ch concentrations (0.5%–3%). Also, a decrease in shoot and root length was observed at 1%–3% Ch concentrations. Under such conditions, the highest and lowest amount of root dry weight was obtained with 0.2% and 3% Ch concentrations, respectively (Table 1).

At −1.2 MPa osmotic potential, application of low concentrations of Ch increased germination percentage and germination index and rate. The highest amount of shoot length, root length, and root dry weight was observed at 0.05% Ch treatment; shoot dry weight was the highest at 0.4% Ch treatment, however (Table 1).

No shoot length was recorded at high concentrations of Ch and −1.2 MPa osmotic potential (Table 1), so dry weights, proline and MDA content, protein, POX, and CAT activities were not calculable.

Proline accumulation increased at −0.4 and −0.8 MPa osmotic potentials without chitosan treatment as compared with the control (0.0 MPa) (Figure 1). In the case of unstressed seedlings, the highest and lowest proline contents were obtained at 0.2% and 3% Ch concentrations, respectively. At −0.8 MPa osmotic potential, proline content was decreased by low concentrations of Ch relative to the control, whereas it was increased by high concentrations of Ch.

Malondialdehyde content was increased at −0.4 and −0.8 MPa osmotic potentials compared with the control (0.0 MPa) (Figure 2). Under unstressed conditions, application of low concentrations of Ch decreased MDA content relative to the control when no chitosan was applied. In addition,

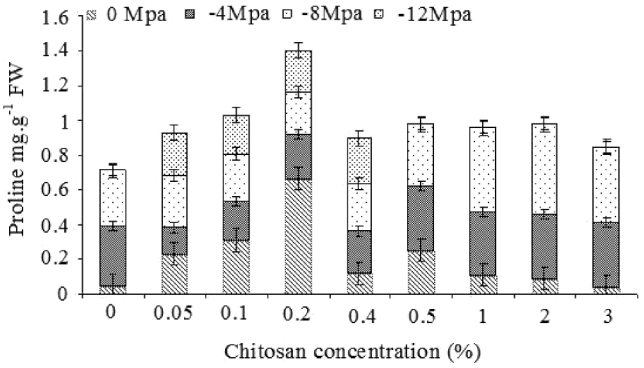

FIGURE 1 Effect of different chitosan concentrations and water stress levels on proline content in safflower seedlings. Standard errors indicate significant differences with control groups at $P < 0.05$.

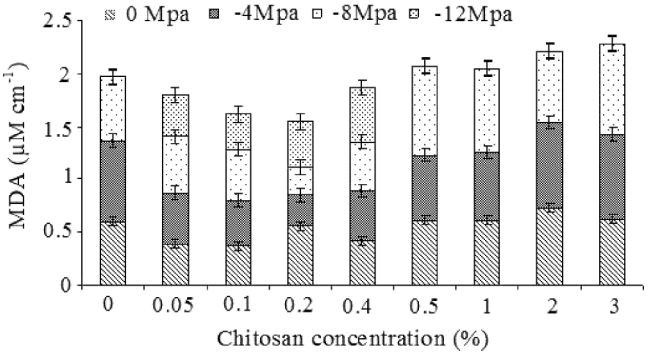

FIGURE 2 Effect of different chitosan concentrations and water stress levels on MDA content in safflower seedlings. Standard errors indicate significant differences with control groups at $P < 0.05$.

the stressed seeds treated with low concentrations of Ch had a lower MDA content in their seedlings. At −0.8 MPa osmotic potential, application of high concentrations of Ch increased MDA accumulation over the control treatment.

The protein content of stressed seedlings that were not treated with chitosan was low (Figure 3). In addition, application of the lowest concentrations of Ch increased protein content in the stressed seedlings, whereas the value of this trait was decreased by 1% to 3% Ch treatments.

Catalase activity increased in response to low osmotic potential (Figure 4). Low concentrations of Ch increased CAT activity over the control treatment in the unstressed seedlings. At −0.4 MPa osmotic potential there were no significant differences in CAT activity among different concentrations of chitosan. At −0.8 MPa osmotic potential seeds treated with

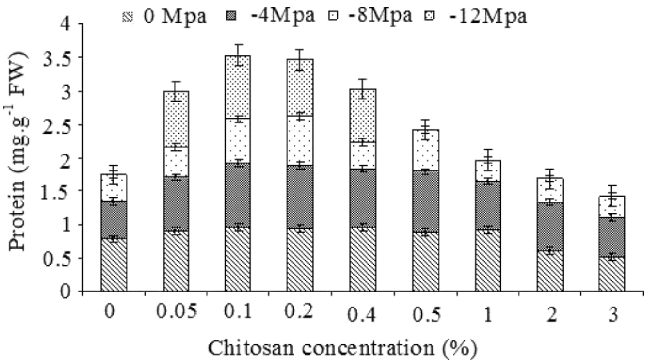

FIGURE 3 Effect of different chitosan concentrations and water stress levels on protein content in safflower seedlings. Standard errors indicate significant differences with control groups at $P < 0.05$.

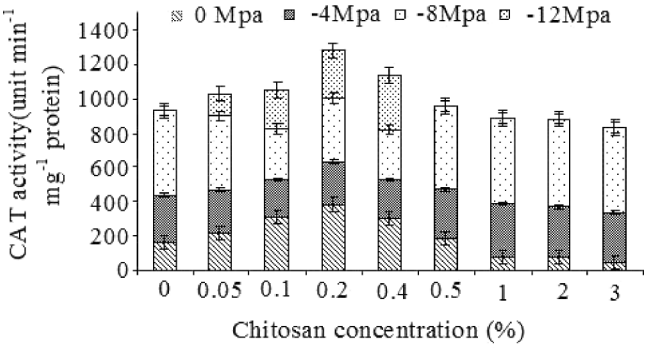

FIGURE 4 Effect of different chitosan concentrations and water stress levels on CAT activity in safflower seedlings. Standard errors indicate significant differences with control groups at $P < 0.05$.

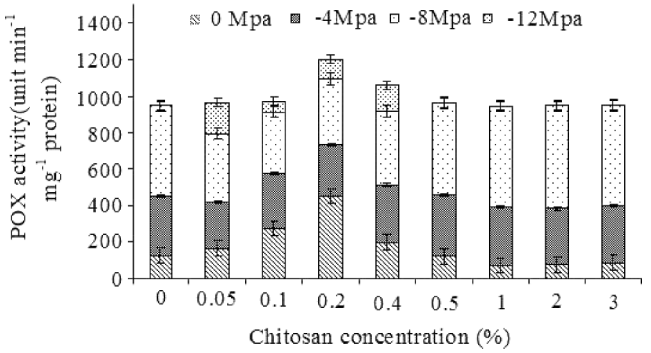

FIGURE 5 Effect of different chitosan concentrations and water stress levels on POX activity in safflower seedlings. Standard errors indicate significant differences with control groups at $P < 0.05$.

low concentrations of Ch caused a significant decrease in CAT activity in seedlings compared with the control treatment.

The POX activity was higher in water-deficit-stressed seedlings than in non-stressed seedlings (Figure 5). Unstressed seeds treated with low concentrations of Ch had more POX activity in their seedlings, whereas when seeds were exposed to −0.4 MPa osmotic potential, Ch treatment had no effect on POX activity in seedlings. Peroxidase activity decreased at −0.8 MPa osmotic potential at low Ch concentrations relative to the control.

DISCUSSION

Results indicated that germination percentage, germination index and rate, shoot and root length, and their dry weight decreased with reductions in water potential. Under unstressed conditions, different concentrations of

chitosan did not have any effect on germination percentage, whereas germination percentage increased with increasing concentrations of Ch up to 0.4% in stressed seedlings. This difference may be related to the differences in antioxidant enzyme activities, proline concentration, and plasma membrane permeability under these two conditions. It seems that priming with chitosan reduced detrimental effects of water stress. Our results are in agreement with the findings of Ruan and Xue (2002), who showed that chitosan accelerates seed germination and improves the tolerance of hybrid rice seedlings to stress. Also, we found that germination percentage decreased at high chitosan concentrations (0.5%–3%) under stress conditions. It seems that treatment of seeds with relatively high concentrations of chitosan might have caused an absorptive obstruction of water because of the stickiness of chitosan. Furthermore, the high concentration of chitosan might be toxic for seeds.

Accumulation of organic compounds, such as proline, in the cytoplasm plays an important role in osmotic adjustment in plants. This molecule is strongly hydrophilic and alleviates stress damage in plant cells by reducing the water potential. In this study, proline content in safflower seedlings increased because of water deficit. In stressed seedlings, low concentrations of Ch (0.05%–0.4%) caused a decrease in proline content, whereas the reverse was true at high concentrations of proline, i.e., increased levels of proline were observed at high chitosan concentrations. In this study, priming with chitosan improved osmotic tolerance in safflower seeds.

The MDA content increased when water deficit was increased to −0.8 MPa. By contrast, we observed that MDA content decreased at low concentrations of chitosan whether or not seedlings were stressed. Water stress caused an increase in ROS, which led to oxidative damage to the membrane system of plants. Cooperation of protective enzymes, such as POD and CAT, could eliminate ROS and keep a homeostatic balance between production and cleaning of ROS and reduce the level of free radicals. It seems that chitosan at low concentrations can prevent lipid oxidation and increase malondialdehyde by suppressing free radicals directly or through antioxidant enzymes.

According to our results, water deficit decreased protein content. Application of 0.05%–0.5% concentrations of chitosan increased protein content in seedlings at high osmotic potential (−0.8 MPa). In the current study, the activities of CAT and POX increased with increasing stress level to −0.8 MPa. It seems that CAT and POX enzymes played an important role in preserving safflower seedlings against water-deficit stress. Treated seeds with 0.05%–0.5% chitosan concentrations had more CAT activity than the control treatment (unstressed seedlings). However, treatment of seeds with different concentrations of chitosan had no effect on POX activity. The present experiment indicated that when safflower seedlings were subjected to osmotic potential of −0.8 MPa, POX and CAT activity decreased because of low chitosan concentrations. This reduction might be caused

by the superoxide scavenging ability provided by low concentrations of chitosan because antioxidants play an important role in preventing stress-induced accumulation of toxic concentrations of reactive oxygen species. This suggested that the antioxidant properties of chitosan could also enhance resistance to oxidative stress in plants. In this study, we also showed that use of an exogenous substance, such as chitosan, could mitigate the effects of water stress and increase the oxidation resistance of plants under prolonged drought conditions.

The results showed that low concentrations of chitosan significantly reduced the harmful effects of water deficit on germination of seedlings. In addition, chitosan could overcome severe or prolonged drought through the reduction of enzyme activity caused by scavenging of reactive oxygen species; thus, seed priming with chitosan improved the speed of germination of safflower seed and improved seedling growth under osmotic potential stress. We concluded that chitosan was an effective biostimulator to enhance seedling growth and plant tolerance to oxidative stress, especially drought stress.

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