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Chitosan effects on physiochemical indicators of drought-induced leaf stress in cowpea

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Abstract

Water deficit stress in crops is associated a damaging oxidative process that is irreversible once it is initiated. This study was conducted to assess the effect of chitosan, a marine polysaccharide with unique bioactive properties that scavenges for reactive oxygen species; hence, chitosan application to plants has been suggested as an aid for reducing oxidative injury caused by water deficit stress. In a greenhouse, potted cowpea plants, *Vigna unguiculata* (L.) Walp. (var. Cream 7), were subject to 12 treatments comprised of 70% (low water deficit stress, control), 50% (moderate stress), and 30% (high stress) field capacity irrigation regimes that were sprayed at the initiation of flowering with 0, 125, 250, and 500 mg/l chitosan. Chitosan application reduced hydrogen peroxide (H_2O_2) accumulation by as much as 37%, lipid peroxidation by as much as 57%, and membrane permeability by up to 16% in leaves from plants under high water deficit stress. The application of chitosan also elevated antioxidant enzyme activities, such as superoxide dismutase by up to 85% and catalase by up to 37%, and accumulations of ascorbic acid, calcium, carotenoids, magnesium, and phenolic compounds that were up to 204%, 29%, 193%, 27%, and 83%, respectively, over leaves of nonsprayed mature plants. Under moderate and severe water deficit, chitosan, particularly at a concentration of 250 mg/liter water, decreased physiochemical indicators of drought stress in leaves, and increased indicators of stress reduction. The greater levels of antioxidants and low H_2O_2 concentration in the chitosan-treated cowpea leaves suggest that chitosan delays effects of water deficit stress. Chitosan might be useful for crop production in situations where water availability is limited, and to make crop plants less attractive to pests that are favored by water deficit stressed plants.

Keywords: antioxidant; lipid peroxidation; oxidative stress; *Vigna unguiculata*; water deficit.
Abbreviations: EC_electrical conductivity.

Introduction

Permanent or temporary water deficit stress limits the growth and distribution of cultivated plants more than any other environmental factors (Ludlow and Mu-Chow, 1990; Shao et al., 2009; Showler, 2012). Water deficit stress occurs when the availability of water is insufficient to maintain plant growth, photosynthesis, and transpiration (Fan et al., 2006), often stunting vegetative growth, inducing flower abortion, (Cothren, 1999; Pimentel, 2004) and promoting oxidative stress associated with leaf senescence (Pinheiro et al., 2004; Farouk and Amany, 2012). Drought-associated changes in plant biochemical processes can reduce photosynthetic CO_2 fixation (Pinheiro et al., 2004) and photosynthetic electron transport while increasing production of reactive oxygen species (Reddy et al., 2004) that cause lipid peroxidation and associated injury to membranes, proteins, and nucleic acids (Gao et al., 2008). Leaf injury from water deficit stress is correlated with vulnerability to oxidative stress, accompanied by chlorophyll loss, decreased soluble protein content, changes in the ratio of chlorophyll a:b (Munne-Bosch, 2007; Farouk, 2011) and it is related to an increase in reactive oxygen species, lipid peroxidation, and membrane leakage (Navabpour et al., 2003).

To mitigate such oxidative damage caused by reactive oxygen species, plants have developed an antioxidative system involving antioxidants and antioxidative enzymes such as ascorbic acid, α-tocopherol, proline, carotenoids, phenol, superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase (Zabalza et al., 2007). Superoxide dismutase, for example, is a major scavenger of reactive oxygen species that produces hydrogen peroxide (H_2O_2) and oxygen. The H_2O_2 is scavenged by glutathione reductase, and several classes of peroxidases (e.g., ascorbate peroxide) which catalyze reduction of H_2O_2 to water and oxygen (Noctor and Foyer, 1998; Bray et al., 2000). The activity of biochemicals allows short-term acclimation to temporary water deficit, but these antioxidants and enzymes cannot overcome the effects of extreme or prolonged drought.

Chitosan is a cationic polysaccharide produced by alkaline N-deacetylation of chitin. Beneficial effects of chitosan in enhancing tolerance of plants to biotic and abiotic stresses crop plants, and its relevance to agriculture, have been described (Farouk et al., 2011; Farouk and Amany, 2012). Antioxidant activity of chitosan has also been suggested (Park et al., 2004)

because it can scavenge OH and O_2^- radicals and because it has DNA-protective properties (Prashanth et al., 2007). Treatment of waterthyme, *Hydrilla verticillata* (L. f.) Royle, with chitosan increases the activity of superoxide dismutase and decreases malondialdehyde (a product of lipid peroxidation caused by reactive oxygen species) concentrations (Xu et al., 2007) while scavenging superoxide anions (Yin et al., 2002; Sun et al., 2004).

Although the antioxidant activity of chitosan is largely known for its biomedical, nutritional, and environmental protection aspects (Sun et al., 2007; Xu et al., 2007; Meng et al., 2008), there is little understanding of chitosan's role in reducing drought-associated leaf damage. The purpose of this study is to assess effects of chitosan applied as a foliar spray on selected reactive oxygen species and antioxidants, and on antioxidant enzyme activities in cowpea leaf tissue under different levels of water deficit stress.

Results

Treatment effects on soluble indicators of water deficit stress

Calcium. In leaf tissue from the 50% (moderate water deficit stress) and 30% (high stress) field capacity irrigation regimes, Ca^{++} levels were 16% and 32% lower than in the 70% (low stress) field capacity control leaf tissue (Table 1). The leaves sprayed with 125 mg/l chitosan had 12% and 21% less Ca^{++} in the moderate and high stress regimes than in the low stress regime; leaves treated with 250 mg/l of chitosan had 7% and 22% less; and leaves sprayed with 500 mg/l of chitosan were not different in terms of Ca^{++} accumulation in the low and moderate stress regimes, but the high stress regime had 16% less Ca^{++} than the low stress regime (Table 1).

Under low stress conditions, Ca^{++} in cowpea leaf tissue increased 16% and 35% when 125 and 250 mg/l of chitosan, respectively, was applied, but no effect was detected in leaves of plants sprayed with 500 mg/l of chitosan (Table 1). In the moderate stress regime, 125 and 250 mg/l of chitosan resulted in Ca^{++} concentration increases of 22% and 50%, respectively, but at 500 mg/l of chitosan, Ca^{++} increased by only 14% (Table 1). In the high stress regime, Ca^{++} concentrations in plants treated with 125 and 250 mg/l of chitosan were elevated by 13% and 29%, respectively, but plants sprayed with 500 mg/l chitosan showed an increase of only 9% (Table 1).

Magnesium. As water deficit conditions increased from low to moderate and high in the absence of chitosan, leaf Mg^{++} concentrations declined by 9% and 18%, respectively (Table 1). In leaves sprayed with 125 mg/l of chitosan, Mg^{++} levels were 10% and 22% greater in the low and moderate stress regimes, respectively. The high stress regime, however, was associated with 15% and 11% less Mg^{++}, where 250 and 500 mg/l of chitosan, respectively, was applied compared against leaves from the low stress regime (Table 1).

Leaves in the low stress regime accumulated 11% and 22% more Mg^{++} when leaves were treated with 125 and 250 mg/l of chitosan, respectively, than leaves treated with 500 mg/l of chitosan (Table 1). In the moderate stress regime, leaves sprayed with 125 and 250 mg/l of chitosan accumulated 10% and 29%, respectively, more Mg^{++} than nonsprayed leaves, and in the high stress regime, leaves sprayed with the same concentrations of chitosan had 11% and 27%, respectively, more Mg^{++} than nonsprayed leaves (Table 1). At each of the three water deficit stress levels, the highest rate of chitosan failed to alter Mg^{++} concentrations (Table 1).

Soluble protein. In the absence of chitosan application, soluble protein concentrations declined by 10% and 21% in the moderate and high stress regimes, respectively, as compared with the low stress regime (Table 1); soluble protein reduction was 4% and 15%, respectively, at the 125 mg/l chitosan rate; and 8% and 10%, respectively, at the 250 mg/l chitosan rate. Although no difference was detected between the low and moderate stress regimes in leaves of plants sprayed with 500 mg/l of chitosan, soluble protein was 12% lower in the high stress regime than in the low stress regime (Table 1).

Under well-watered conditions, 125 and 250 mg/l of chitosan increased soluble protein concentrations in leaf tissue by 4% and 13%, respectively, over nonsprayed leaves (Table 1). In the pots of the moderate stress regime, 125 and 250 mg/l of chitosan was associated with 12% and 17% greater soluble protein concentrations, respectively, over nonsprayed leaves, and in the high stress regime soluble protein was 13% and 29% more abundant than in nonsprayed leaves (Table 1). At the 500 mg/l chitosan rate, however, soluble protein levels were not affected in the low stress regime, and increased in the moderate and high stress regimes by only 7% and 9%, respectively (Table 1).

Treatment effects on reactive oxygen species

Hydrogen peroxide. In plants that were not sprayed with chitosan, reductions in water availability from the low stress regime to the moderate and high stress regimes resulted in 21% and 31% greater concentrations of H_2O_2, respectively (Table 2). In leaves of plants sprayed with 125 mg/l of chitosan, the same reductions in water availability were associated with 20% and 35% more H_2O_2, respectively; 250 mg/l of chitosan caused an increase of 16% in the high stress regime, and 500 mg/l of chitosan caused increases of 19% and 24%, respectively (Table 2).

H_2O_2 concentrations in leaves from the low stress regime declined by 22%, 30%, and 8% in association with 125, 250, and 500 mg/l chitosan sprays, respectively, compared against leaves of nonsprayed plants (Table 2). In leaves of the moderate stress regime, H_2O_2 levels fell by 24%, 38%, and 9%, respectively (Table 2), and in the high stress regime, H_2O_2 decreased by 26%, 37%, and 13% in response to the three respective rates of chitosan compared to nonsprayed leaves (Table 2).

Treatment effects on physiochemical indicators of injury

Membrane permeability. In cowpea plants that were not sprayed with chitosan, water deficit differences between the low stress regime and the moderate and high stress regimes were associated with 9% and 10% increases, respectively, in membrane permeability (Table 2). In plants sprayed with 125 mg/l of chitosan, leaf membrane permeability increased by 6% and 12%, respectively; by 13% and 19%, respectively, in leaves of plants sprayed with 250 mg/l of chitosan; and by 10% and 12%, respectively, in leaves of plants that were sprayed with 500 mg/l of chitosan (Table 2).

Percentage membrane permeability in cowpea plants grown under low stress conditions was reduced in association with 125, 250, and 500 mg/l chitosan sprays by 8%, 22%, and 4%,

Table 1. Mean (±SE) concentrations (mg/g dry leaf tissue weight) of soluble protein, calcium, and magnesium from 80-d-old cowpea plants under different levels of water deficit and rates of chitosan application.

Treatments		Soluble protein	Calcium (Ca^{++})	Magnesium (Mg^{++})
Water deficit stress[a]	Chitosan (mg/l)			
Low	None	6.10 ± 0.02 cd	1.24 ± 0.02 de	0.45 ± 0.003 cd
	125	6.35 ± 0.02 b	1.44 ± 0.03 c	0.50 ± 0.022 b
	250	6.89 ± 0.04 a	1.67 ± 0.02 a	0.55 ± 0.004 a
	500	5.98 ± 0.01 de	1.22 ± 0.01 def	0.44 ± 0.003 cd
Moderate	None	5.46 ± 0.09 f	1.04 ± 0.01 g	0.41 ± 0.005 ef
	125	6.12 ± 0.01 cd	1.27 ± 0.03 de	0.45 ± 0.003 cd
	250	6.37 ± 0.02 b	1.56 ± 0.08 b	0.53 ± 0.006 ab
	500	5.86 ± 0.10 e	1.19 ± 0.01 ef	0.44 ± 0.004 de
High	None	4.82 ± 0.08 h	0.84 ± 0.03 h	0.37 ± 0.004 g
	125	5.44 ± 0.06 f	1.14 ± 0.02 f	0.41 ± 0.011 ef
	250	6.22 ± 0.03 bc	1.31 ± 0.01 d	0.47 ± 0.017 c
	500	5.27 ± 0.08 g	1.03 ± 0.03 g	0.39 ± 0.006 fg
F		99.990	59.622	34.065
P		<0.0001	<0.0001	<0.0001

Means within columns followed by different letters are significantly different ($P < 0.05$); df for each analysis was 11, 35. [a]Low (control), 70% field capacity irrigation; moderate, 50% field capacity irrigation; high, 30% field capacity irrigation.

respectively, compared against nonsprayed leaves (Table 2). Under moderate stress conditions, the three concentrations of chitosan spray reduced membrane permeability by 10%, 19%, and 4%, respectively; and under conditions of high stress, membrane permeability decreased by 6% and 16%, but the 500 mg/l concentration of chitosan did not alter membrane permeability (Table 2).

Lipid peroxidation. As water availability in the three controls declined from low stress to conditions of moderate and high stress, lipid peroxidation increased by 82% and 105%, respectively (Table 2). In cowpea plants were sprayed with 125 mg/l of chitosan, lipid peroxidation increased by 29% in response to high stress, but water deficit effects on lipid peroxidation were not detected in plants sprayed with 250 mg/l of chitosan. The moderate and high stress regimes were associated with 66% and 88%, respectively, greater lipid peroxidation in plants sprayed with 500 mg/l of chitosan than in plants of the low stress regime (Table 2).

Lipid peroxidation in nonsprayed plants grown in the low stress regime declined by 10%, 14%, and 7% in association with chitosan sprayed at concentrations of 125, 250, and 500 mg/l, respectively (Table 2). In the moderate stress regime, leaf lipid peroxidation was reduced by 47%, 51%, and 15%, respectively, in comparison with nonsprayed leaves, and in the high stress regime, leaf lipid peroxidation declined by 43%, 57%, and 15%, respectively (Table 2).

Treatment effects on detoxifying agents

Superoxide dismutase. Compared with leaves of plants grown under low water deficit conditions, moderate, and high water deficit stress resulted in reduced superoxide dismutase activity by 22% and 11%, respectively, where chitosan was not applied (Table 3). In contrast, in leaves of plants sprayed with 125 mg/l chitosan, superoxide dismutase activity increased by 27% under conditions of high stress compared against plants grown under low stress conditions (Table 3). The 250 mg/l rate of chitosan, however, caused a 9% reduction in superoxide dismutase activity where water deficit stress was moderate, and a 14% increase in leaves of plants grown under high stress conditions leaves (Table 3). The 500 mg/l chitosan spray increased the enzyme's activity by 15% in leaves of plants grown under conditions of high stress (Table 3).

Superoxide dismutase activity in plants grown under low stress conditions increased by 27% and 45% as a result of 125 and 250 mg/l chitosan sprays, respectively, in comparison with nonsprayed leaves, but 500 mg/l of chitosan had no effect (Table 3). In moderately stressed plants, superoxide dismutase activity was elevated by 66%, 68%, and 26% in response to the three respective chitosan concentrations compared with nonsprayed leaves and more highly stressed leaves had 81%, 85%, and 37% more activity than nonsprayed leaves (Table 3).

Catalase. Moderate, and high levels of stress were associated with catalase activity reductions of 6% and 14%, respectively, compared to leaves of plants grown under low stress conditions (Table 3). Among plants sprayed with 125 mg/l of chitosan, catalase activity was elevated in moderately stressed plants by 5%, but it was reduced in the high stress regime by 19%. Among plants sprayed with 250 mg/l of chitosan, moderate stress was associated with a 4% increase in the enzyme's activity while the high stress regime elicited a 22% decline (Table 3). Although plants sprayed with 500 mg/l of chitosan did not show a change in catalase activity from low to moderately stressed leaves, higher stress caused a decline of 25% from activity where stress was low (Table 3).

Catalase activity in cowpea plants grown in the low stress regime increased by 35%, 50%, and 30% in plants sprayed with 125, 250, and 500 mg/l chitosan, respectively, compared against nonsprayed leaves (Table 3). In leaves of moderately stressed plants, catalase activity increased by 51%, 67%, and 38%, respectively, over the nonsprayed leaves; and under high stress, increases were 27%, 37%, and 14%, respectively (Table 3).

Peroxidase. In the absence of chitosan, decreasing water availability from the low stress regime to the moderate and high stress regimes was associated with 15% and 27% greater

Table 2. Mean (±SE) H_2O_2 (µM/g fresh leaf tissue weight), percentage membrane permeability, and lipid peroxidation (µM/g fresh leaf tissue weight) in leaf tissue from 80-d-old cowpea plants under different levels of water deficit and rates of chitosan application.

Treatments		Hydrogen peroxide (H_2O_2)	Membrane permeability	Lipid peroxidation
Water deficit stress[a]	Chitosan (mg/l)			
Low	None	19.3 ± 0.3 e	79.2 ± 0.6 c	7.2 ± 0.3 e
	125	14.9 ± 0.4 gh	73.0 ± 0.3 e	6.5 ± 0.1 f
	250	13.6 ± 0.3 h	61.7 ± 1.6 g	6.2 ± 0.1 f
	500	17.8 ± 0.1 f	75.7 ± 0.6 d	6.7 ± 0.1 ef
Moderate	None	23.4 ± 0.7 b	86.7 ± 0.4 a	13.1 ± 0.4 b
	125	17.9 ± 0.5 f	77.6 ± 0.6 cd	6.9 ± 0.1 ef
	250	14.5 ± 0.1 h	69.8 ± 1.1 f	6.4 ± 0.1 ef
	500	21.2 ± 0.1 cd	83.0 ± 0.5 b	11.1 ± 0.6 c
High	None	25.2 ± 0.4 a	87.1 ± 0.1 a	14.8 ± 0.1 a
	125	20.0 ± 0.5 e	81.6 ± 0.6 b	8.3 ± 0.7 d
	250	15.8 ± 0.6 g	73.4 ± 0.3 e	6.5 ± 0.1 ef
	500	22.0 ± 0.4 c	85.1 ± 0.3 a	12.6 ± 0.1 b
F		75.809	120.645	119.644
P		<0.0001	<0.0001	<0.0001

Means within columns followed by different letters are significantly different ($P < 0.05$); df for each analysis was 11, 35. [a]Low (control), 70% field capacity irrigation; moderate, 50% field capacity irrigation; high, 30% field capacity irrigation.

peroxidase activity, respectively (Table 3). Leaves sprayed with 125 mg/l of chitosan had 10% and 19% greater peroxidase activity, respectively, as water was reduced; by 25% and 24%, respectively, in leaves sprayed with 250 mg/l of chitosan; and by 7% in the high stress regime in leaves sprayed with 500 mg/l of chitosan (Table 3).

Peroxidase activity in leaves from the low stress regime declined by 14%, 29%, and 12% when sprayed with 125, 250, and 500 mg/l of chitosan, respectively, compared with nonsprayed leaves (Table 3). In the moderate stress regime, peroxidase activity was reduced by 18%, 23%, and 22%, respectively, and in the high stress regime, reductions were 20%, 31%, and 26%, respectively, below activity levels detected in nonsprayed leaves (Table 3).

Polyphenol oxidase. Reductions in irrigation from the low to moderate and high stress regimes in the absence of chitosan caused increases of 51% and 83%, respectively, in terms of polyphenol oxidase activity (Table 3). In leaves of plants that were sprayed with 125 mg/l of chitosan, reducing water availability from the low stress to moderate and high stress regimes was associated with 36% and 77% greater polyphenol oxidase activity, respectively; in plants sprayed with 250 mg/l of chitosan, polyphenol oxidase activity increased by 32% and 31%, respectively; and 500 mg/l of chitosan was associated with 36% and 50% increases, respectively (Table 3).

Polyphenol oxidase activity in cowpea leaves grown under low stress conditions decreased by 8%, 25%, and 15% when sprayed with 125, 250, and 500 mg/l of chitosan, respectively, below the activity found in nonsprayed leaves (Table 3). The same rates of chitosan in the moderate stress regime were associated with 16%, 35%, and 23% declines, respectively, and in the high stress regime, with 11%, 47%, and 30% declines, respectively (Table 3).

Treatment effects on protectants

Carotenoids. In nonsprayed plants, carotenoids in cowpea leaf tissue declined by 65% with the reduction of water availability from low to high stress conditions (Table 4). In plants sprayed with 125 mg/l of chitosan, carotenoid content declined by 45% and 63% when water deficit stress was moderate and high, respectively; at 250 mg/l chitosan, reductions were 18% and 57%, respectively, and at 500 mg/l chitosan, carotenoid levels declined by 27% and 56%, respectively (Table 4).

In plants grown in the low stress regime, 125 and 250 mg/l chitosan sprays increased carotenoid content of leaf tissue by 72% and 135%, respectively, over corresponding nonsprayed leaf tissue, but 500 mg/l of chitosan had no effect (Table 4). In the moderate and high stress regimes, carotenoid content increased by 154% and 193%, respectively, in response to 250 mg/l chitosan, but the 125 and 500 mg/l chitosan sprays did not affect carotenoid levels (Table 4).

Ascorbic acid. In leaves of cowpea plants that were not sprayed with chitosan, reduction of water availability from low to moderate and high water deficit stress resulted in 18% and 44% less ascorbic acid, respectively (Table 4). In plants sprayed with 125 and 250 mg/l of chitosan, ascorbic acid levels were reduced by 33% and 65%, respectively, but only when stress increased from low to high (Table 4). At the highest chitosan spray concentration, ascorbic acid accumulations were 7% and 35% less in the moderate and high stress regimes, respectively (Table 4).

Ascorbic acid concentrations in cowpea leaves grown in the low stress regime were 9% and 13% greater when 125 and 250 mg/l of chitosan, respectively, were sprayed compared with nonsprayed plants, but 500 mg/l of chitosan had no effect (Table 4). In the moderate stress regime, the level of ascorbic acid increased 27%, 36%, and 9% where chitosan was applied at 125, 250, and 500 mg/l, respectively, over nonsprayed leaves (Table 4). Where water deficit stress was high, the 125, 250, and 500 mg/l chitosan sprays caused ascorbic acid concentrations to rise by 86%, 204%, and 44%, respectively, above nonsprayed leaves (Table 4).

Phenolic compounds. In nonsprayed plants where water availability was reduced from low stress to moderate and high stress conditions, phenolic compound accumulations declined

Table 3. Mean (±SE) superoxide dismutase, catalase, peroxidase, and polyphenole oxidase (activity/g fresh leaf tissue weight/h) in leaf tissue from 80-d-old cowpea plants under different levels of water deficit and rates of chitosan application.

Treatments		Superoxide dismutase	Catalase	Peroxidase	Polyphenole oxidase
Water deficit stress[a]	Chitosan (mg/l)				
Low	None	58.3 ± 1.1 e	47.7 ± 0.8 g	442.0 ± 4.9 c	41.8 ± 0.3 f
	125	73.9 ± 0.8 cd	64.3 ± 1.7 d	379.8 ± 4.3 f	38.6 ± 0.6 g
	250	84.5 ± 0.5 b	71.5 ± 1.1 b	313.4 ± 4.8 g	31.2 ± 0.1 i
	500	61.9 ± 1.1 e	62.8 ± 0.2 d	390.4 ± 5.1 ef	35.7 ± 0.4 h
Moderate	None	45.7 ± 2.1 g	44.6 ± 0.5 b	510.1 ± 3.8 b	63.0 ± 0.2 c
	125	75.7 ± 1.1 cd	67.2 ± 0.7 c	416.4 ± 0.7 d	52.6 ± 1.2 d
	250	76.8 ± 0.9 c	74.5 ± 0.6 a	391.9 ± 2.2 ef	41.1 ± 0.4 f
	500	57.8 ± 2.7 e	61.7 ± 0.7 d	398.7 ± 4.1 e	48.6 ± 0.5 e
High	None	52.1 ± 1.0 f	41.0 ± 0.4 i	563.0 ± 4.7 a	76.7 ± 0.3 a
	125	94.1 ± 1.7 a	51.9 ± 0.4 f	453.0 ± 6.1 cd	68.3 ± 0.6 b
	250	96.2 ± 1.4 a	56.1 ± 1.6 e	388.1 ± 2.0 ef	41.0 ± 0.3 f
	500	71.2 ± 3.4 d	46.8 ± 1.6 gh	416.7 ± 1.9 d	53.4 ± 0.5 d
F		89.079	127.370	258.051	687.090
P		<0.0001	<0.0001	<0.0001	<0.0001

Means within columns followed by different letters are significantly different ($P < 0.05$); df for each analysis was 11, 35. [a]Low (control), 70% field capacity irrigation; moderate, 50% field capacity irrigation; high, 30% field capacity irrigation.

by 27% and 43%, respectively (Table 4). In plants sprayed with 125 mg/l of chitosan, the same reductions of water availability were associated with 4% and 39% declines of phenolics concentrations, respectively; in plants sprayed with 250 mg/l of chitosan, reductions were 0.8% and 5%, respectively; and 12% and 34%, respectively, in plants sprayed with 500 mg/l of chitosan (Table 4).

In the low stress regime, cowpea plants sprayed with 125 and 250 mg/l of chitosan had 7% and 11% more phenolic compounds, respectively, than in leaves from nonsprayed plants, but 500 mg/l of chitosan had no effect (Table 4). Cowpea plants in the moderately stressed regime had 40%, 50%, and 16% more phenolic compounds in plants sprayed with 125, 250, and 500 mg/l of chitosan, respectively, than in nonsprayed leaves (Table 4). Where water was least available, the 125 and 250 mg/l chitosan sprays increased phenolics by 14% and 83%, respectively, over nonsprayed leaves from the high stress regime, but in plants sprayed with 500 mg/l chitosan, only 7% more phenolic compounds were detected than in nonsprayed leaves (Table 4).

Discussion

The reduction in Ca^{++} in the water deficit stressed cowpea plants is known to be related to leaf senescence (Leidi et al., 1991). Mg^{++} is implicated in the regulation of protein synthesis (Flowers and Dalmond, 1992), hence, its decline was observed along with soluble protein in the drought-stressed cowpea leaves, and Leidi et al. (1991) determined that low Mg^{++} was also responsible for decreased leaf chlorophyll content.

Drought-induced pre-senescence in cowpea leaves is accompanied by the increased production of some reactive oxygen species found in our study, such as H_2O_2, superoxide, and its more toxic derivative hydroxyl radical (Breusegem and Dat, 2006). These reactive oxygen species oxidize proteins, lipids, and DNA when they reach certain threshold levels associated with nutrient relocation to developing pods (before plant mortality occurs) resulting in the heightened lipid peroxidation we found in cowpea leaf tissue, and later cellular damage and death that is responsible for senescence (Kukavica and Jovanovic, 2004). Leaf senescence is commonly characterized by the decreased soluble protein we found in our study, relatively low chlorophyll concentration which is associated with declining Mg^{++} (Leidi et al., 1991), and by the observed increases in membrane permeability because of heightened lipid peroxidation occurring in cell membranes (Farouk, 2011). Among the different reactive oxygen species, only H_2O_2 is relatively stable and able to penetrate the plasma membrane in an unaltered form (Hung et al., 2005). In addition to being toxic to chloroplasts and being powerful inhibitors of the Calvin cycle, H_2O_2 is regarded as a signal molecule with a regulatory role in gene expression (Hung et al., 2005). The most deleterious effect of H_2O_2 under water deficit conditions is that, at relatively high concentrations, it can trigger genetically programmed cell mortality, and our study indicates that, in cowpea, drought stress induced elevated H_2O_2 production and lipid peroxidation that induce oxidative injury. As a strong oxidant, H_2O_2 can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity which promotes injury, including leaf senescence. It also changes the redox status of surrounding cells where it initiates an antioxidative response by acting as a signal of oxidative stress (Sairam and Srivastava, 2000). The loss in functionality and integrity of cell membranes because of lipid peroxidation in cowpea has also been reported to occur in wheat plants, *Triticum aestivum* L., where lipid peroxidation increases in senescing tissues accompanied by increased electrolyte leakage (Farouk, 2011). Such damage can result from various mechanisms including oxidation and cross-linkage of protein thiols, inhibition of key membrane proteins as H^+-ATPase, and changes to the composition and fluidity of membrane lipids (Farouk, 2011). The increased membrane permeability found in association with water deficit in cowpea plants is a likely consequence of the intensified lipid peroxidation. Further, ascorbic acid, a non-enzymatic antioxidant and free radical scavenger, occurred in lesser amounts in the cowpea leaves grown under drought conditions, reducing the level of protection from reactive oxygen species,

Table 4. Mean (±SE) concentrations of carotenoids and ascorbic acid (mg/g fresh leaf tissue weight), and phenolic compounds (mg catecol/100 g fresh leaf tissue weight) from 80-d-old cowpea plants under different levels of water deficit and rates of chitosan application.

Treatments		Carotenoids	Ascorbic acid	Phenolics
Water deficit stress[a]	Chitosan (mg/l)			
Low	None	0.162 ± 0.010 c	16.3 ± 0.1 cd	21.7 ± 0.1 ef
	125	0.278 ± 0.001 b	17.7 ± 0.1 ab	23.2 ± 0.1 bc
	250	0.380 ± 0.050 a	18.4 ± 0.1 a	24.0 ± 0.1 a
	500	0.176 ± 0.032 c	15.8 ± 0.1 d	21.1 ± 0.3 f
Moderate	None	0.123 ± 0.003 cde	13.4 ± 0.5 f	15.9 ± 0.5 h
	125	0.154 ± 0.003 cd	17.0 ± 0.2 be	22.2 ± 0.1 de
	250	0.313 ± 0.008 b	18.2 ± 0.2 a	23.8 ± 0.1 bc
	500	0.129 ± 0.004 cde	14.7 ± 0.5 e	18.5 ± 0.5 g
High	None	0.056 ± 0.005 f	9.1 ± 0.2 i	12.4 ± 0.2 k
	125	0.104 ± 0.007 def	11.9 ± 0.2 g	14.1 ± 0.2 i
	250	0.164 ± 0.001 c	17.2 ± 0.1 b	22.7 ± 0.1 cd
	500	0.078 ± 0.006 ef	10.3 ± 0.3 b	13.3 ± 0.2 j
F		30.026	141.032	274.419
P		<0.0001	<0.0001	<0.0001

Means within columns followed by different letters are significantly different ($P < 0.05$); df for each analysis was 11, 35. [a]Low (control), 70% field capacity irrigation; moderate, 50% field capacity irrigation; high, 30% field capacity irrigation.

an association that has also been reported in leaves of corn, *Zea mays* L. (Prochazkova et al., 2001; Farouk, 2011), and exogenous application of ascorbic acid to wheat plants stimulates growth and development, presumably because it decreases levels of reactive oxygen species (Malik and Ashraf, 2012). Although peroxidase activity increases during times of drought stress in certain plants (Klar et al., 2006), some antioxidizing enzymes measured in our study, such as peroxidase and polyphenole oxidase, did not consistently decline decreases as water availability declined, lower superoxide dismutase activity likely also contributed to the heightened concentrations of H_2O_2 under increasing levels of water deficit.

Defensive mechanisms against oxidative injury affiliated with drought stress, including production of superoxide dismutase, catalase, and peroxidase (which decompose superoxide radicals and H_2O_2), can be bolstered by exogenous application of chitosan (Mittler, 2002; Farouk and Amany, 2012). Several reports have indicated that chitosan can have beneficial effects on plant survival under conditions of water deficit stress, often by causing the closure of stomata which conserves water (Bittelli et al., 2001), usually in association with the inhibition of reactive oxygen species formation (Alia et al., 1993; Halliwell et al., 1995; Xie et al., 2001). The lower level of lipid peroxidation in plants sprayed with chitosan, whether under well watered or drought conditions, suggests that chitosan protects against oxidative damage. Antioxidant properties of chitosan are mainly attributable to its abundant active hydroxyl and amino groups that react with reactive oxygen species to form stable and relatively nontoxic macromolecular radicals (Xie et al., 2001). It is likely that chitosan activates superoxide dismutase and catalase involved in the detoxification of H_2O_2 in plants. Moreover, our study demonstrates that application of chitosan increased carotenoids, ascorbic acid, and total phenolic content in cowpea leaf tissue, decreasing the generation of free radicals and lipid peroxidation when plants are stressed (Alia et al., 1993; Malik and Ashraf, 2012). In this regard, phenolic compounds, for example, inhibit the oxidation of lipids and proteins by the transfer of phenolic hydrogen atoms to a radical (Burton et al., 1985; Halliwell et al., 1995; Mayer et al., 2002; Jang et al., 2007).

Protection from antioxidants involves compounds such as carotenoids, ascorbic acid, phenolic compounds, proline, and an enzymatic system including superoxide dismutase, catalase, peroxidase, and the Halliwell-Asada pathway (Foyer et al., 1994). Carotenoids, for example, are involved in the protection of the photosynthetic apparatus against photo-inhibitory damage by singlet oxygen ($^1O_2$) produced by the excited triplet state of chlorophyll (Foyer and Harbinson, 1994). Catalase, in cooperation with peroxidases and other enzymes, destroys the H_2O_2 produced by superoxide dismutase and other catalyzed reactions (Bowler et al., 1992; Foyer et al., 1994). Because superoxide dismutase and catalase activities increased in our study as a result of chitosan application under both well watered and drought stress conditions, we propose that superoxide dismutase and catalase play important roles in the defensive responses of plant cells to oxidative stress (Zabalza et al., 2007). The greater inhibition of antioxidant enzymes under drought stress as compared to antioxidant-treated plants indicates increased inactivation of antioxidant enzymes by reactive oxygen species (Djanaguiaman et al., 2005) probably because of toxic effects associated with the higher turnover rate of H_2O_2 and other harmful reactive oxygen species that impair enzyme activities (Noctor and Foyer, 1998). Our findings suggest that chitosan-sprayed plants can eliminate reactive oxygen species through induction of higher superoxide dismutase and catalase activities as indicated by the greater availability of phenolic compounds. Application of chitosan elevates total chlorophyll content by stimulating its biosynthesis and by delaying its degradation (Chibu and Shibayama, 2001). Increased chlorophyll in connection with chitosan application, however, might also be attributed to greater amounts of Mg^{++} (Leidi et al., 1991) detected in our study, and to efficient scavenging of reactive oxygen species that would have otherwise destroyed the chlorophyll by antioxidant enzymes and antioxidants. Chloroplasts are a major source of reactive oxygen species production in plants, but the organelles lack catalase to scavenge reactive oxygen species; hence, ascorbic

acid, which increased in response to chitosan, must act as a substrate for ascorbate peroxidase for scavenging reactive oxygen species produced in the thylakoid membranes (Davey et al., 2000).

The decline in protection against water deficit stress in the cowpea plants sprayed with 500 mg/l of chitosan might have resulted from factors that occurred alone or in combination. Reports have indicated that relatively high rates of chitosan can halt or reduce growth of plant shoots and roots (Young et al., 1982; Asghari-Zakaria et al., 2009; Sheikha and Al-Malki, 2011), both of which could decrease plant vigor and the rates of biochemical processes. Other studies have demonstrated that high rates of chitosan can induce mortality of leaf epidermal cells by the destruction of their nuclei (Vasil'ev et al., 2009), reduce photosynthesis and chlorophyll content (Sheikha and Al-Malki, 2011; Mondal et al., 2012), and increase cell permeability (Young et al., 1982) and production of reactive oxygen species (Khokon et al., 2010). High chitosan rates have also been associated with declines in K, P, N, and sugars (Abdel-Mawgoud et al., 2010) and immobilization of enzymes including catalase, peroxidase, and superoxide dismutase (Çetinus and Oztop, 2000; Galovic et al., 2002; Bindhu and Abraham, 2003; Dutta et al., 2004; Çetinus et al., 2007). However, we were unable to identify the precise mechanism for the lower efficacy of the highest rate of chitosan used in our study.

Materials and Methods

Experimental setup and design

This study was conducted using potted cowpea plants, *Vigna unguiculata* (L.) Walp. (var. Cream), at the Agricultural Botany Department, Mansoura University, Mansoura, Egypt, during 2008. Cowpea seeds were obtained from the Legume Research Institute, Ministry of Agriculture, Giza, Egypt, and sterilized with 1.5% chlorox, washed three times with distilled water, and coated with N-fixing okadeen (*Rhizobia*) procured from the General Organization for Agriculture Equalization Fund, Ministry of Agriculture, Cairo, Egypt. Sowing occurred on 10 April in 7.6-liter plastic pots, each containing 25 kg of air dried soil (80% sand, 15.5% silt, and 4.5% clay; pH, 7.8). Phosphorus (P) and potassium (K) fertilizers were added to the soil before sowing at a rate of 5 g P_2SO_5 per pot (0.02% by mass) in the form of calcium super phosphate and 2 g K_2O per pot (0.008% by mass) in the form of potassium sulfate. After sowing, irrigation was applied to saturate the soil at 2-d intervals until the young plants reached the fourth leaf stage; at that time the plants were thinned to seven per pot. Ammonium nitrate (33.5%) was added at a rate of 4 g N per pot at the seedling stage and again when the plants began to flower.

Pots of cowpea plants were assigned to one of 12 treatments arranged in a completely randomized experimental design, each treatment replicated three times. Fifteen days after the seeds were planted, 12 pots were irrigated to 70% field capacity (considered as being well watered and plants grown in those pots were under low water deficit stress), 12 to 50% field capacity (moderately stressed), and 12 to 30% (highly stressed) field capacity. The cowpea foliage of three pots within each of those irrigation regimes were sprayed with chitosan at 125 mg/l to runoff with a hand-held pressure pump sprayer when the plants began to flower; three were sprayed with chitosan at 250 mg/l, and three with 500 mg/l. Each of the chitosan sprays included 0.5% Tween 20 (Cayman Chemical, Ann Arbor, Michigan, USA). The three pots in each irrigation regime were sprayed with water, each considered to be the nontreated control for that particular irrigation regime. Pots were weighed individually every 2 d and weight reductions were assumed to represent water losses from evaporation and transpiration; the amount of water lost each 2 d was replaced to maintain the assigned field capacities. Three uniform plants were uprooted from each pot at the pre-senescent full bloom stage (80 d after planting) for Ca, Mg, and biochemical analyses.

All data were analyzed using one-way ANOVA and means were separated using Tukey's HSD ($P \leq 0.05$) (CoHort Software, 2008). Data were not transformed before analysis because normality and homogeneity of variance assumptions were not violated.

Soluble indicators of water deficit stress

Ca^{++} and Mg^{++} concentrations were measured using versenate methods (Richard, 1984). Soluble protein concentration was measured at 595 nm using bovine serum albumin as a standard according to the method of Bradford (1976). For ion content, dry leaf samples were digested with $HClO_3$ H_2SO_4 until the solution was clear, cooled, and brought to a volume of 50 ml using deionized water.

Reactive oxygen species

Lipid peroxidation was estimated as thiobarbituric acid reactive substances (*i.e.*, malondialdehyde) measured as µmoles/g of leaf tissue fresh weight (Shao et al., 2005). Malondialdehyde content was calculated using an extinction coefficient of 155 nM/cm.

Physiochemical indicators of injury

Percentage electrolyte leakage measurements were applied to assess membrane permeability according to the method of Goncalves et al. (2007) using an electrical conductivity meter (Hanna Instruments, Bedfordshire, England). Electrical conductivity of the resulting solution (EC_1) was recorded after incubation. Samples were then placed in a boiling water bath for 30 min, cooled to room temperature, and a second reading was recorded (EC_2). Electrolyte leakage was calculated as EC_1/EC_2 and expressed as a percentage. H_2O_2 content was estimated by forming a titanium-hydro peroxide complex (Rao et al., 1997).

Detoxifying agents

Antioxidant enzymes were extracted using the method of Mukherjee and Choudhuri (1983). Superoxide dismutase activity was determined according to Dhinda et al. (1981), and catalase was assayed by measuring the disappearance of H_2O_2 (Teranishi et al., 1974). Peroxidase activity was determined using the method of Reuveni and Reuveni (1995) and polyphenol oxidase activity was measured using the method of Kar and Mishra (1976). The activities of each of the enzymes were expressed as activity/g fresh weight/h.

Protectants

Carotenoid was extracted for 24 h at room temperature in methanol after adding traces of sodium carbonate. Carotenoid concentrations were determined spectrophotometrically (Spekol 1300, Analytik, Jena, Denmark) according to Lichtenthaler and Wellburn (1983). Ascorbic acid was extracted from plant material and titrated using 2,6-dichlorophenol indophenole as described by Sadasivani and Manickam (1996). Total phenolic compounds were determined using Folin-Ciocalteau reagent according to the method of Singleton and Rossi (1965).

Conclusions

Chitosan sprays at the low and moderate rates used in this study can improve the capacity of cowpea plants to survive under water deficit stress from the inhibition of leaf physiochemicals and processes indicative of drought stress and enhancing physiochemicals and processes that protect against water deficit-associated leaf injury. We suggest that chitosan might be useful for several purposes, including the maintenance of crop productivity where water is a limiting factor, and possibly for reducing water deficit stress interactions that favor infestation by some pests (Showler and Castro, 2010; Showler, 2012).

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