

Mannose-Induced Modulations in Antioxidants, Protease Activity, Lipid Peroxidation, and Total Phenolics in Etiolated Wheat Leaves

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Abstract Modulation of different antioxidants, total phenolics, lipid peroxidation, and protease activity as a result of mannose treatment (1%) was studied in leaves of etiolated wheat seedlings. Changes in these biochemicals were monitored up to 96 h after treatment at 24-h intervals. Mannose treatment induced a significant increase in protease activity throughout the scanning period, coupled with a gradual decrease in leaf protein content. Membrane lipid peroxidation (MDA content) was higher at 24 and 72 h after treatment. MDA content remained higher for a longer period due to mannose treatment. During the initial 24 h of mannose treatment, only catalase and total phenolic contents were increased. Catalase activity was down regulated with increasing duration of treatment. On the other hand, peroxidase (POD, APX) activities were initially unaffected but increased with increasing treatment duration. The decreased level of lipid peroxidation at 96 h may be due to detoxification of H₂O₂ by peroxidases. Superoxide dismutase activity was not affected by mannose treatment. In conclusion, evidence is provided that mannose can modulate the expression of the enzymatic antioxidant defense system in wheat leaves.

Keywords D-mannose · Catalase · Peroxidase · Superoxide dismutase · MDA · ROS

Introduction

Mannose (Man) has been used as a tool to study the role of orthophosphate and was often described to be toxic (Herold and Lewis 1977). At the plant or tissue level, Man was shown to inhibit the growth of shoots, roots, stems, and pollen tubes (Herold and Lewis 1977), inhibit germination via a hexokinase-mediated step (Pego and others 1999), and induce leaf wilting (Herold and Lewis 1977). At the cell level, Man has been shown to cause an imbalance of glucose and ion uptake (Herold and Lewis 1977), respiration, ATP synthesis (Garrard and Humphreys 1969), photosynthesis (Harris and others 1983), starch synthesis, and degradation (Herold and Lewis 1977), and to induce cell apoptosis (Stein and Hansen 1999).

Mannose is readily taken up by roots and converted to Man-6-P by the action of hexokinase (Stein and Hansen 1999). However, Man-6-P is not utilized further because of a deficiency of Man-6-P isomerase, which is necessary for its conversion to Fru-6-P (Goldsworthy and Street 1965). The primary cause of most of the inhibitory effects of Man is the accumulation of Man-6-P (M6P) because of the absence or low activity of Man phosphate isomerase (Herold and Lewis 1977). This accumulation results in a strong Pi sequestration, which leads to a decreased synthesis of ATP and a subsequent imbalance of metabolism (Herold and Lewis 1977). The irreversible formation of Man-6-P inhibits respiration by depleting cells of the orthophosphate required for ATP production (Goldsworthy and Street 1965).

The high accumulation of Man-6-P inhibits phospho-Glc isomerase, thus blocking glycolysis (Goldsworthy and Street 1965). Severely impaired primary metabolism with a dramatic drop in glycolytic intermediates, respiration rate, endopeptidase activities, and adenine nucleotide content as a result of sequestration of Pi under M6P has been reported

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in maize root tips (Brouquisse and others 2001). In addition to these metabolic effects, Man and other hexoses repress the transcription of genes required in photosynthesis and the glyoxylate cycle (Jang and Sheen 1994, 1997). The specificity of the D- over the L-enantiomer indicates that Man toxicity is not the result of osmotic stress (Stein and Hansen 1999). Rather, the effect of Man could be the result of interference with Glc utilization and phosphate availability (Goldsworthy and Street 1965).

In the present work, the effect of D-mannose on anti-oxidants (SOD, CAT, APX, POD, and ascorbate) and total phenolics was investigated in etiolated wheat leaves. In parallel, changes in protein content, proteolytic activities, and membrane lipid peroxidation were also followed. Changes in these biochemicals were monitored up to 96 h after mannose treatment at 24-h intervals. Interdependence and relationships among these biochemicals under D-mannose treatment are also discussed.

Material and Methods

Uniform seeds (44.05 ± 3.07 mg) of wheat (PAK-81) were selected for the experiment. Seeds in three replicates (35 seedlings per replicate) were germinated in darkness for 24 h at $25 \pm 1^\circ\text{C}$ on wet filter paper in Petri dishes. Germinated seeds were then covered with a lid to minimize evaporation, and growth continued in an incubator for 24 h at $25 \pm 1^\circ\text{C}$. Mannose treatment was applied on the fourth day of germination. Except for controls, water as the medium was replaced with 1% (equal to 56 mM) mannose solution and the growth of the seedlings was continued at $25 \pm 1^\circ\text{C}$ for 96 h. Previously, a similar concentration of mannose had been reported to induce toxicity leading to cell death and DNA laddering in *Arabidopsis* (Stein and Hansen 1999). Initial leaf samples were collected after every 24 h (24, 48, 72, and 96 h) and used for different biochemical analyses. For growth response studies, seedling fresh and dry weights and shoot length and shoot dry weight were measured after 96 h of D-mannose treatment.

Extraction of Antioxidant Enzymes

For extraction of enzymes, fresh leaves (0.5 g) were ground in extraction buffer specific for different enzymes and centrifuged at 15,000g for 20 min at 4°C . The supernatant was separated and used for the determination of different enzyme activities.

Superoxide Dismutase

Samples (0.5 g) were homogenized in extraction buffer consisting of 50 mM potassium phosphate, pH 7.8, 0.1%

(w/v) BSA, 0.1% (w/v) ascorbate, and 0.05% (w/v) β -mercaptoethanol as described by Dixit and others (2001). The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). The reaction solution (3 ml) contained 50 μM NBT, 1.3 μM riboflavin, 13 mM methionine, 75 nM EDTA, 50 mM potassium phosphate buffer (pH 7.8), and 20–50 μl enzyme extract. The test tubes containing the reaction solution were irradiated under a light (15-W fluorescent lamps) at $78 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 15 min. The absorbance of the irradiated solution at 560 nm was determined with a spectrophotometer (Hitachi U-2800, Tokyo, Japan). One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT.

Catalase and Peroxidase

For the estimation of catalase (CAT) and peroxidase (POD), leaves (0.5 g) were homogenized in medium composed of 50 mM potassium phosphate buffer (pH 7.0) and 1 mM dithiothreitol (DTT). Activities of POD and CAT were measured using the method of Chance and Maehly (1955) with some modification. For measurement of POD activity, the assay solution (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 40 mM H_2O_2 , and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. The increase in absorbance of the reaction solution at 470 nm was recorded after every 20 s. One unit of POD activity was defined as an absorbance change of $0.01 \text{ unit min}^{-1}$. For measurement of CAT activity, the assay solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2 , and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. The decrease in absorbance of the reaction solution at 240 nm was recorded after every 20 s. An absorbance change of $0.01 \text{ unit min}^{-1}$ was defined as 1 unit of CAT activity. Enzyme activities were expressed on a protein basis. Protein concentration of the enzyme extract was measured by the dye binding assay as described by Bradford (1976).

Ascorbate Peroxidase

For the estimation of ascorbate peroxidase (APX) activity, 0.5-g plant samples were extracted in 2.5 ml homogenizing medium containing 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM ascorbate, and 2% (v/v) β -mercaptoethanol (Dixit and others 2001). For assay of the enzyme activity, the rate of hydrogen peroxide-dependent oxidation of ascorbic acid was determined in a reaction mixture that contained 50 mM potassium

phosphate buffer (pH 7.0), 0.6 mM ascorbic acid, and enzyme extract (Chen and Asada 1989). The reaction was initiated by addition of 10 μ l 10% (v/v) H₂O₂, and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm for 3 min.

Protease Activity

For estimation of protease activity, samples were extracted in 50 mM potassium phosphate buffer (pH 7.8). Protease activity was determined by the casein digestion assay described by Drapeau (1974). A series of tubes were equilibrated with 2.0 ml of 1% casein at 37°C for 5 min. To all the tubes, 100 μ l of protease extract was added and mixed well. A reagent blank was also included. Exactly 10 min after adding the sample, the reaction was stopped by adding 2.0 ml TCA solution and mixing well. Tubes were then allowed to stand for 10 min and then the reaction solution was filtered to remove the precipitate formed during the reaction. The absorbance of filtrate was measured at 280 nm. By this method 1 unit was defined as the amount of enzyme that releases acid-soluble fragments equivalent to 0.001 A₂₈₀ min⁻¹ at 37°C and pH 7.8. Enzyme activity was expressed on a protein basis.

Malondialdehyde Content

The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA, a product of lipid peroxidation) content determined by the thiobarbituric acid (TBA) reaction using the method of Heath and Packer (1968), with minor modifications as described by Dhindsa and others (1981) and Zhang and Kirkham (1994). A 0.25-g leaf sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10,000g for 5 min. To a 1-ml aliquot of the supernatant, 4 ml 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifuging at 10,000g for 10 min, the absorbance of the supernatant at 532 nm was read and the value for the nonspecific absorption at 600 nm was subtracted. The MDA content was calculated by using the extinction coefficient of 155 mM⁻¹ cm⁻¹.

Total Phenolic Content

A microcolorimetric method described by Ainsworth and Gillespie (2007) was used for total phenolic assay, which utilizes Folin-Ciocalteu (F-C) reagent. A standard curve was prepared using different concentrations of gallic acid and a linear regression equation was calculated. A 0.2-g leaf sample was homogenized in 0.8 ml ice cold 95% methanol using an ice cold mortar and pestle. The samples

were then incubated at room temperature for 48 h in the dark and then centrifuged at 10,000g for 5 min at room temperature. The supernatant was removed and used for TPC measurement. One hundred microliters of supernatant were mixed with 100 μ l of 10% (v/v) F-C reagent, vortexed thoroughly, and then 800 μ l of 700 mM Na₂CO₃ were added. Samples were then incubated at room temperature for 2 h. Blank corrected absorbance of samples was measured at 765 nm. Phenolic content (gallic acid equivalent) of samples was determined using a linear regression equation.

Ascorbic Acid (Vitamin C)

For ascorbic acid determination, the 2,6-dichloroindophenol (DCIP) method described by Hameed and others (2005), which measures only reduced ascorbic acid, was used. Briefly, each molecule of vitamin C converts a molecule of DCIP into a molecule of DCIPH₂, and that conversion can be monitored as a decrease in the absorbance at 520 nm. A standard curve was prepared using a series of known ascorbic acid concentrations. A simple linear regression equation was calculated to find the ascorbate concentration in unknown samples.

Statistical Analysis

Significance of data was tested by analysis of variance and Duncan's multiple range test at $p < 0.05$ and where applicable at $p < 0.01$ using MSTAT software. Values presented in graphs are mean \pm standard deviation (SD).

Results

The growth inhibitory effect of mannose on plants is well documented. The seedling growth response was compared after 96 h of D-mannose treatment (Fig. 1). Mannose treatment decreased seedling fresh and dry weights compared to those of nontreated controls. This decrease in seedling dry weight was also reflected in reduced shoot dry weight. Shoot length was also reduced as a result of mannose treatment. This shows that mannose treatment induced growth retardation with less biomass accumulation compared to control. Protein degradation and enhanced proteolytic activities are among the consequences of several abiotic and biotic stress conditions. Mannose induced a significant increase ($p < 0.001$) in protease activity throughout the scanning period (24–96 h) (Fig. 2a). Maximum protease activity was observed at 72 h after mannose treatment. Under nontreated conditions, protease activity remained unchanged at all time intervals (24–96 h). Leaf protein content increased ($p < 0.01$) only at 48 h after

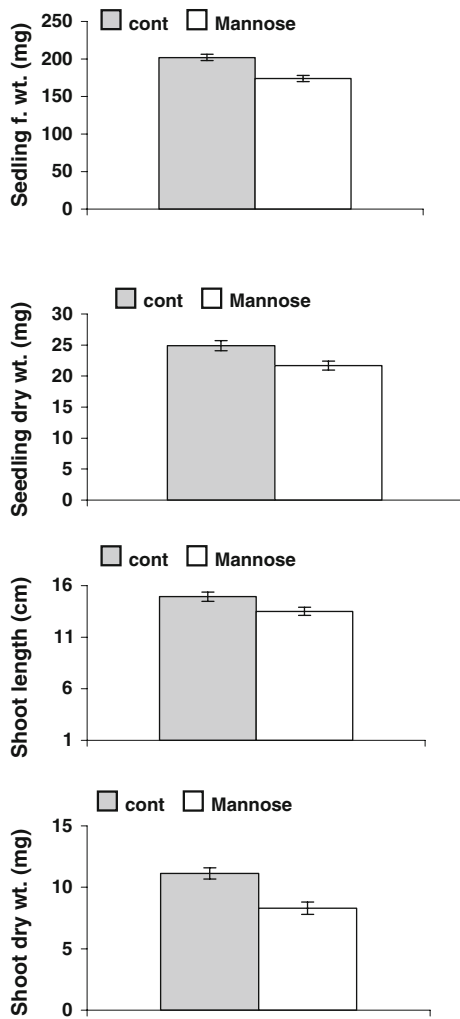


Fig. 1 Growth response of etiolated seedlings compared after 96 h of D-mannose treatment. Gray bars indicate control and white bars indicate 1% D-mannose-treated samples. Each data point represents the mean of three samples \pm SD

mannose treatment (Fig. 2b), whereas it remained unchanged at other time intervals. Under nontreated conditions, a gradual decrease in protein content was observed up to 72 h. Moreover, mannose treatment decreased ($p < 0.05$) the soluble protein content when the level at 72 h after treatment was compared with the level at 24 h after treatment.

Peroxidation of membrane lipids in terms of MDA content significantly ($p < 0.05$) increased at 24 and 72 h ($p < 0.01$) after mannose treatment (Fig. 3a). However, MDA content remained unchanged at other time intervals (48 and 96 h) compared with controls. Under nontreated conditions, a peak in lipid peroxidation was observed at 48 h but decreased gradually afterward. In the case of mannose treatment, the peak in lipid peroxidation was observed at 72 h instead of at 48 h. This indicates that mannose treatment prolonged the duration of higher MDA

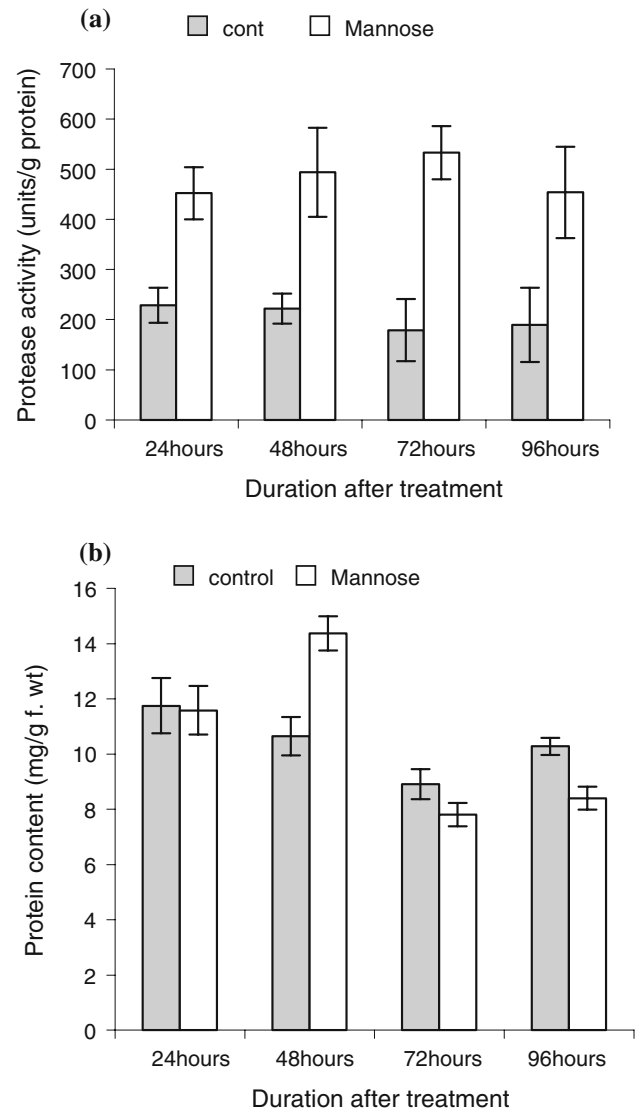


Fig. 2 Protease activity (a) and protein content (b) in wheat (*Triticum aestivum* L.) leaves at different intervals after mannose treatment. Gray bars indicate control and white bars indicate 1% D-mannose-treated samples. Each data point represents the mean of three samples \pm SD

content by at least 24 h. MDA and TPC levels rose more rapidly with mannose treatment compared with control leaves and also remained at elevated levels for a longer period of time. Under nontreated conditions, the highest level (peak) of phenolics was observed at 72 h and then the level decreased at 96 h (Fig. 3b). Mannose treatment increased the TPC to peak level (highest level under the untreated condition) just after 24 h of treatment and then it remained higher and unchanged until 72 h. This indicates that mannose treatment prolonged the duration of the higher phenolic content.

Catalase activity increased significantly ($p < 0.001$) after 24 h of treatment. Catalase activity was down

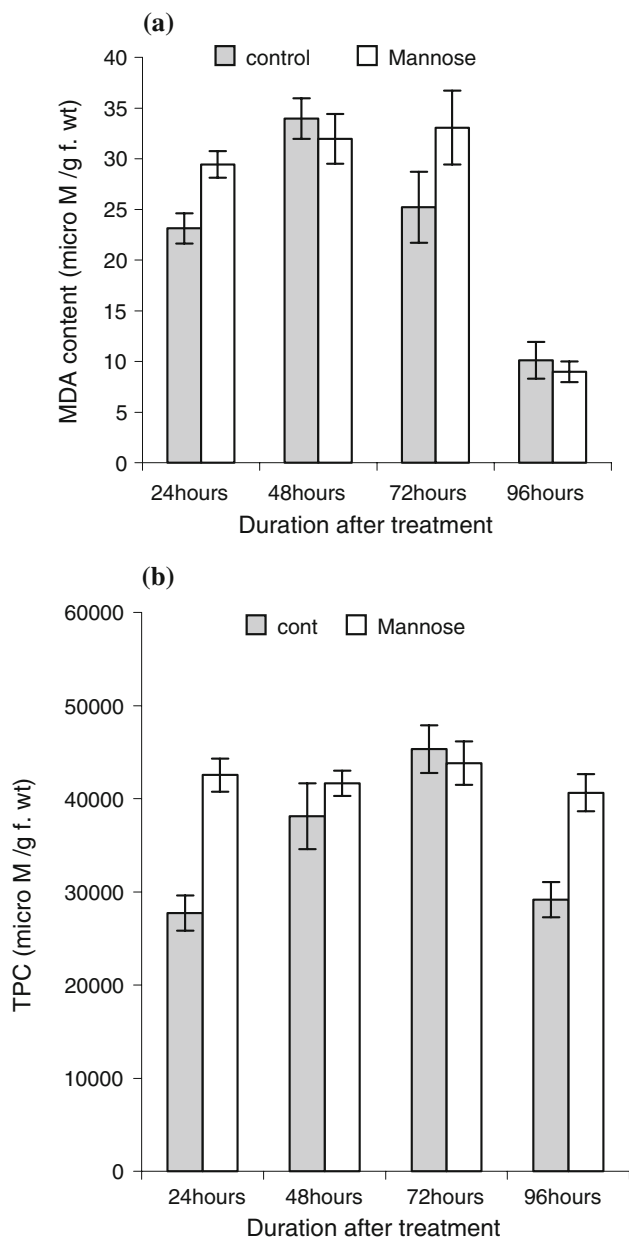


Fig. 3 Malondialdehyde (MDA) (a) and total phenolic content (b) in wheat (*Triticum aestivum* L.) leaves at different intervals after mannose treatment. Gray bars indicate control and white bars indicate 1% D-mannose-treated samples. Each data point represents the mean of three samples \pm SD

regulated with increasing duration of stress and was significantly ($p < 0.01$) lower at other time intervals compared with that of controls (Fig. 4a). Except at 24 h, an overlapping trend was observed for catalase activity in nontreated and treated conditions; however, the magnitude of activity was lower in treated conditions.

Contrary to catalase, peroxidase activity initially (24 and 48 h) remained unaffected although significantly ($p < 0.001$) increased with increasing stress duration

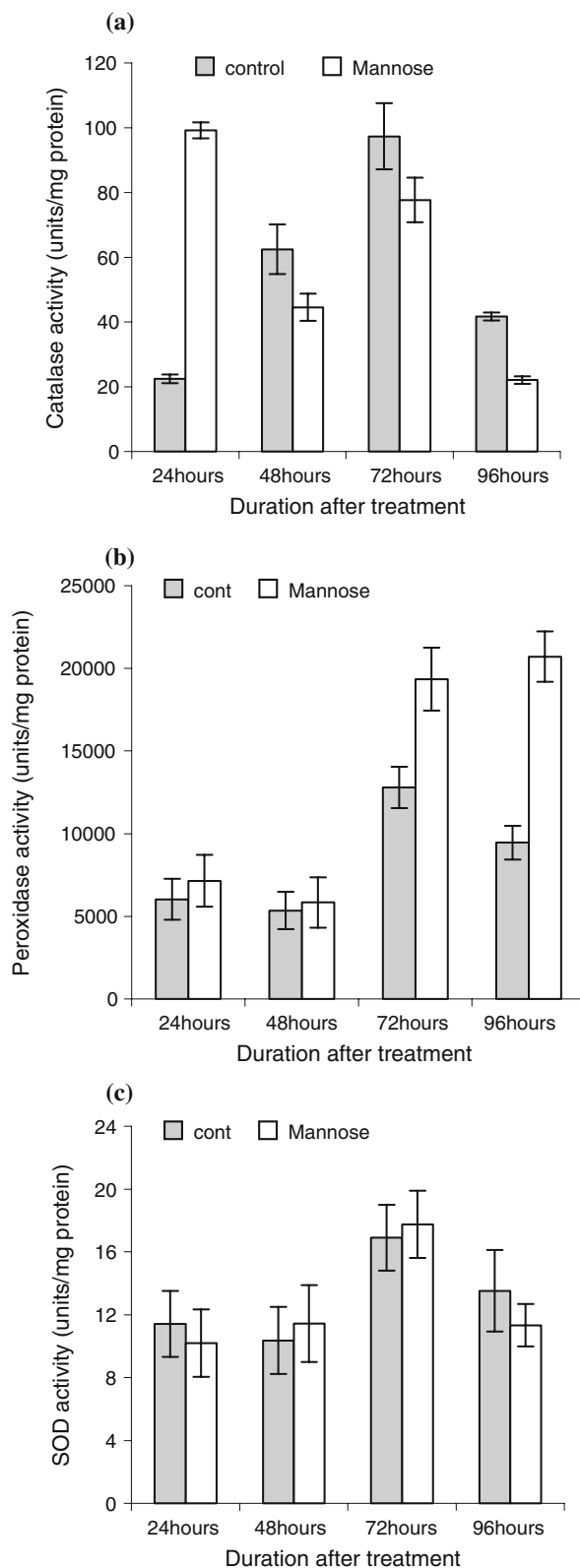


Fig. 4 Activities of catalase (a), peroxidase (b), and superoxide dismutase (c) in wheat (*Triticum aestivum* L.) leaves at different intervals after mannose treatment. Gray bars indicate control and white bars indicate 1% D-mannose-treated samples. Each data point represents the mean of three samples \pm SD

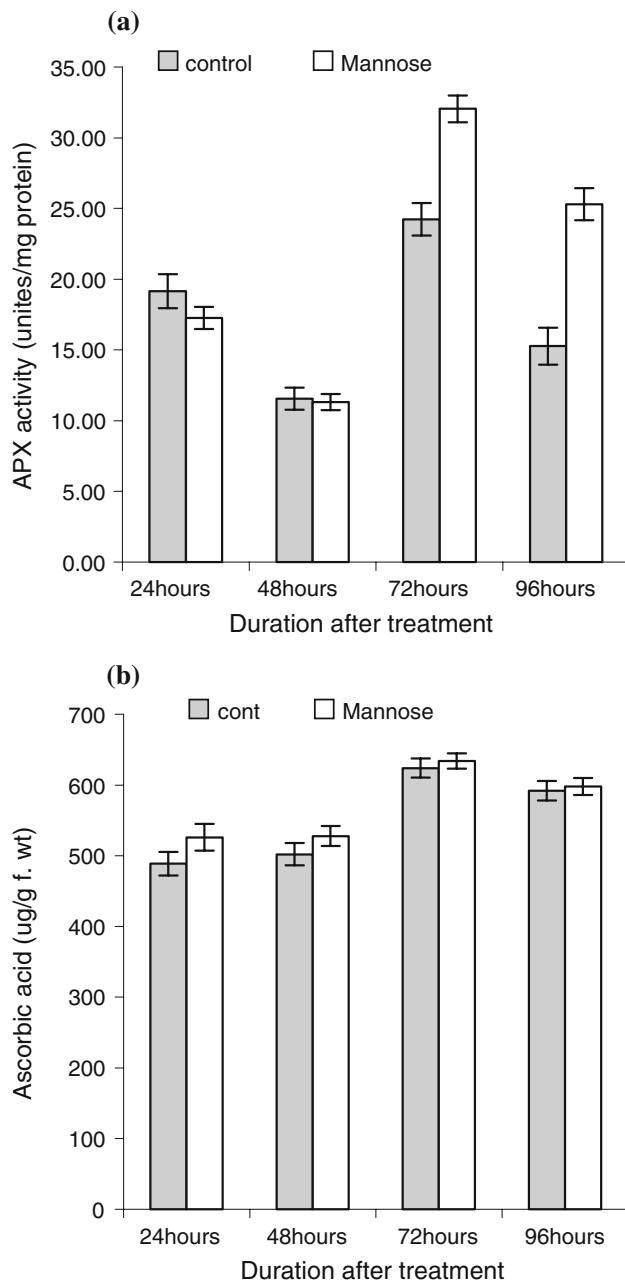


Fig. 5 Ascorbate peroxidase (APX) activity (a) and ascorbic acid content (b) in wheat (*Triticum aestivum* L.) leaves at different intervals after mannose treatment. Gray bars indicate control and white bars indicate 1% D-mannose-treated samples. Each data point represents the mean of three samples \pm SD

(Fig. 4b). With a peak at 72 h under nontreated conditions, peroxidase activity was down regulated at 96 h. However, with mannose treatment, peroxidase activity was increased at 72 h and remained higher until 96 h. This means that mannose treatment raised the peroxidase level during 72–96 h of treatment. Superoxide dismutase (SOD) activity remained unaffected under mannose treatment at all time intervals (Fig. 4c). Throughout the scanning period, similar

trends were observed for SOD activity in treated and nontreated conditions, with peak levels after 72 h.

Similar to peroxidase, ascorbate peroxidase (APX) activity also remained unaffected during the initial 48 h of mannose treatment (Fig. 5a). Ascorbate peroxidase significantly ($p < 0.001$) increased over control after 72 and 96 h of mannose treatment. APX activity peaked at 72 h in treated and nontreated conditions; however, the magnitude was significantly higher in the mannose-treated leaves. Mannose treatment did not significantly modulate the ascorbate content in wheat leaves (Fig. 5b). An overlapping trend was observed under mannose treatment and in nontreated conditions with a relatively higher ascorbate concentration at 72 h compared with that at other time intervals. Ascorbate content was higher between 72 and 96 h compared with the level during the initial 48 h under treated and nontreated conditions.

Discussion

Mannose has been shown to inhibit the growth of shoots, roots, stems, and pollen tubes (Herold and Lewis 1977), inhibit germination via a hexokinase-mediated step (Pego and others 1999), and induce leaf wilting (Herold and Lewis 1977). A similar inhibitory effect of mannose on seedling growth in terms of reduced seedling fresh and dry weights and reduction in shoot length and biomass accumulation was also evident in the present study. This growth inhibitory effect of mannose may be caused by reported cytotoxic effects of mannose due to an imbalance in the mechanisms of glucose and ion uptake (Herold and Lewis 1977), respiration, ATP synthesis (Garrard and Humphreys 1969), photosynthesis (Harris and others 1983), and starch synthesis and degradation (Herold and Lewis 1977).

Protein breakdown and recycling, which depend on the levels of proteolytic enzymes, are essential parts of the plant response to environmental stress (Hieng and others 2004). Proteases are involved in protein maturation, degradation, and protein rebuilding in response to different external stimuli and to removal of abnormal, misfolded proteins (Grudkowska and Zagdanska 2004). Mannose treatment decreased the protein content of leaves when compared before and after prolonged treatment. This decrease in leaf protein content was coupled with a significant increase in leaf protease activity. Previously, a salt stress-induced increase in acid and alkaline proteases, a decrease in protein content, and an increase in free amino acid content have been reported in *B. parviflora* (Parida and others 2002, 2004) and radish (Muthukumarasamy and others 2000). Actually, proteins are hydrolyzed by proteases to release amino acids for storage and/or transport and

for osmotic adjustment during NaCl stress. Our results, indirectly supported by the above-mentioned findings under salt stress, suggest that decreased protein content might be due to increased activity of proteases. Actually, mannose, at concentrations lower than that which trigger apoptosis (Stein and Hansen 1999), inhibits gene expression and biological processes (Brouquisse and others 2001). However, higher mannose concentrations can induce gene expression in plant cells, for example, expression of endonuclease responsible for apoptotic DNA laddering (Stein and Hansen 1999). Even a low mannose concentration (2 mM), along with Pi supply, could increase proteolysis, endopeptidase activities, and root starvation-induced protease (RSIP) expression in maize root tips (Brouquisse and others 2001).

During senescence, the breakdown of membranes due to lipid peroxidation is among one of the factors underlying the increased proteolytic activities (Storey and Beevers 1978). Similarly, in the present study, peroxidation of membrane lipids, along with increased proteolytic activity, was also increased as a result of mannose treatment. Therefore, it seems that breakdown of membranes as a consequence of mannose treatment is also one of the factors responsible for increased proteolytic activity. Enhanced protease activity might occur for proteolysis of proteins released as a result of membrane damage. Proteolysis may serve to release amino acids for synthesis of stress induced/responsive proteins. Other proposed functions of free amino acid accumulation are osmotic adjustment, protection of cellular macromolecules, a form of nitrogen storage, maintenance of cellular pH, detoxification of the cells, and scavenging of free radicals (Parida and others 2004).

Peroxidation of membrane lipids requires active uptake of oxygen. The ability of superoxide radicals, singlet oxygen, H₂O₂, and hydroxyl free radical, collectively called reactive oxygen species (ROS), to initiate lipid peroxidation has been demonstrated (Kellogg and Fridovich 1975; Brennan and Frenkel 1977). Excess ROS triggers phytotoxic reactions such as lipid peroxidation, protein degradation, and DNA mutation (Smirnoff 1993; Alscher and others 1997; McCord 2000). In the present study, peroxidation of membrane lipids was increased at 24 and 72 h after mannose treatment. Mannose treatment also prolonged the duration of higher MDA content. These findings thus provide indirect evidence that excessive ROS were produced after mannose treatment resulting in deterioration of membrane lipids.

Plants detoxify ROS by upregulating antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT). A main role is attributed to SOD in catalyzing the dismutation of superoxide anions to dioxygen and hydrogen peroxide (H₂O₂)

(Raza and others 2007). The increased production of H₂O₂ is subsequently counteracted by peroxidase or catalase (Garratt and others 2002). It is mainly antioxidants that determine the abundance of ROS in the tissue and thereby control the level of lipid peroxidation. Unchanged levels of SOD and peroxidase activities in mannose-treated leaves may have led to uncontrolled ROS production and, in turn, membrane lipid peroxidation. During the initial 24 h only catalase and total phenolic contents were increased, which seems insufficient to completely detoxify the ROS. It has been reported that reduced catalase activity can be compensated by alternative H₂O₂-scavenging mechanisms such as increased ascorbate peroxidase and glutathione peroxidase levels (Willekens and others 1997). Peroxidase and ascorbate peroxidase activities were enhanced between 72 and 96 hours of mannose treatment as an alternate H₂O₂-scavenging mechanism.

Hexoses are known to repress or activate the transcription of genes involved in photosynthesis (Jang and Sheen 1994), glyoxylate metabolism, starch metabolism (Mita and Suzuki-Fujii 1995), pigmentation (Tsukaya and others 1991), and pathogen defense (Herbers and others 1996). In all cases tested, Man is a potent regulator of gene transcription (Jang and Sheen 1994). Data presented in the present study provide evidence that mannose can also modulate the expression of the enzymatic antioxidant defense system in plants.

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