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NEW APOPTOTIC EFFECT OF D-MANNOSE IN WHEAT ROOTS

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Abstract

Effect of D-mannose on antioxidant defense response, apoptotic internucleosomal nuclear DNA (nDNA) degradation, and protease activity was studied in roots of wheat seedlings during early growth. Mannose (1%) induced apoptotic internucleosomal nDNA fragmentation after 96 hours of treatment when it was not observed in control. Superoxide dismutase activity was down regulated parallel to induction of apoptosis. Protease activity was enhanced prior to induction of apoptosis which shows involvement of proteolysis in apoptotic induction process. More over a sharp increase in membrane lipid peroxidation (MDA content) was also observed during apoptosis induction. Catalase and peroxidase activities were increased 24 hours before apoptotic induction while down regulated at time of induction. Total phenolic content was down regulated 48 hours before induction of apoptosis. Collectivity antioxidant defense was down regulated and proteases activities and lipid peroxidation were enhanced during induction of apoptosis and thus play a key role in controlling D-mannose induced apoptotic process. New apoptotic effect of D-mannose in wheat roots in connection with antioxidants and proteases activities is discussed in detail.

Introduction

D-mannose, a safe and readily accessible compound, offers remarkable features for the study of apoptosis in plant cells. The effect of D-mannose on plant cells was studied in two different systems: Arabidopsis roots and maize (*Zea mays*) suspension-cultured cells by Stein & Hansen, (1999). In both systems, exposure to D-mannose was associated with a subset of features characteristic of apoptosis, as assessed by oligonucleosomal fragmentation and microscopy analysis. Furthermore, D-mannose induced the release of cytochrome *c* from mitochondria. Man-induced DNA laddering coincided with the activation of a DNase in maize cytosolic extracts.

The specificity of the D- over the L-enantiomer indicates that D-mannose toxicity is not the result of osmotic stress (Stein & Hansen, 1999). Rather, the effect of D-mannose could be the result of interference with glucose utilization and phosphate availability (Goldsworthy & Street, 1965). D-mannose strongly inhibits root growth and respiration in wheat and tomato (Morgan & Street, 1959). Mannose is readily taken up by roots and converted to mannose-6-P by the action of hexokinase (Stein & Hansen, 1999). However, mannose-6-P is not further utilized due to a deficiency of Man-6-P isomerase, which is necessary for its conversion to Fru-6-P (Goldsworthy & Street, 1965). The primary cause of toxic effects of D-mannose is the accumulation of mannose-6-P (M6P) (Herold & Lewis, 1977) resulting in a strong Pi sequestration, leading to decreased synthesis of ATP and imbalance of metabolism (Herold & Lewis, 1977). A severely impaired primary

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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 in maize root tips (Brouquisse *et al*., 2001)**.** Mannose has also been shown to imbalance ion uptake (Herold & Lewis, 1977), respiration, photosynthesis (Harris *et al.,* 1983), starch synthesis and degradation (Herold & Lewis, 1977) and to induce cell apoptosis (Stein & Hansen, 1999).

Cereal seedlings are unique and very useful model for investigation of apoptosis in plants (Kirnos *et al.,* 1999, Vanyushin 2001, Zamyatnina *et al.,* 2002). Etiolated wheat seedlings have proved to be an excellent model for understanding the apoptosis mechanisms in plant system (Shorning et *al*., 2000; Bakeeva *et al*., 2001; Zamyatnina *et al*., 2003; Vanyushin *et al*; 2002; Vorobjev *et al*., 2005). Firstly, cell death has been found to initiate earlier and to proceed at a faster rate in wheat (Liljeroth & Bryngelsson, 2001). Secondly, the high synchrony of wheat seedling development (Kirnos *et al.,* 1983) makes it possible to investigate the course of apoptosis during the process of plant development. Thirdly, individual organs in cereals are subjected to programmed cell death (organoptosis) (Skulachev 2001). For example coleoptile in cereals function for a relatively short period at the early stages of ontogenesis, and dye quickly as the seedling grows and develops (Zamyatnina *et al.,* 2002).

Using etiolated wheat seedlings as model system in present work, it was investigated that whether D-mannose can influence the apoptotic internucleosomal nDNA fragmentation in wheat roots. Further involvement of enzymatic antioxidants (SOD, CAT, and POD) and total phenolics, in mannose induced cell death was investigated. In parallel, changes in protease activity, soluble proteins and lipid peroxidation were also recorded to understand interactions of these biochemicals under mannose induced apoptosis.

Material and Methods

Uniform seeds (44.05± 3.07mg) of wheat (PAK-81) were selected for experiment. Seeds in three replicates (35 seedlings per replicate) were germinated in darkness for 24 hours at 25±1°C on wet filter paper in petridishes. Germinated seeds were then covered with a lid to minimize the evaporation, and growth was continued in an incubator for 24 h at 25±1°C. Mannose treatment was applied on 4^{th} day of germination. Except for control, water as the medium was replaced with 1% (equal to 56 mM) mannose solution and the growth of the seedlings was continued at 25±1°C for 96 hours. Previously similar concentration of mannose has been reported to induce toxicity leading to cell death and DNA laddering in arabidopsis (Stein & Hansen, 1999). Root samples were collected after every 24 hours (24, 48, 72 and 96 hours) and used for different biochemical analysis. Changes in different biochemicals were monitored up to 96 hours after mannose treatment at 24 hours intervals.

Extraction of antioxidant enzymes: For extraction of enzymes, fresh leaves (0.5 g) were ground in 50mM cold phosphate buffer (pH 7.8) and centrifuged at 15,000×*g* for 20 min at 4 ^{o}C. The supernatant was separated and used for the determination of different enzyme activities.

Superoxide dismutase (SOD): The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Giannopolitis & Ries (1977). The reaction solution (3 ml) contained 50 µM NBT, 1.3µM riboflavin, 13mM methionine, 75nM EDTA, 50mM phosphate buffer (pH 7.8) and 20-50 µl enzyme extract. The test tubes containing the reaction solution were irradiated under a light (15W fluorescent lamps) at 78 $\mu molm^{-2}\ s^{-1}$ 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 which caused 50% inhibition of photochemical reduction of NBT.

Catalase (CAT) and peroxidase (POD): Activities of peroxidase (POD) and catalase (CAT) were measured using the method of Chance & Maehly (1955) with some modification. For measurement of POD activity assay solution (3 ml) contained 50mM phosphate buffer (pH 5.0), 200 mM guaiacol, 400 mM H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Increase in absorbance of the reaction solution at 470 nm was recorded after every 20 s. One unit POD activity was defined as an absorbance change of 0.01 units min^{-1}. For measurement of CAT activity assay solution (3 ml) contained 50mM phosphate buffer (pH 7.0), 59mM H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Decrease in absorbance of the reaction solution at 240 nm was recorded after every 20 s. An absorbance change of 0.01 units min^{-1} was defined as one unit CAT activity. Enzyme activities were expressed on protein basis. Protein concentration of the enzyme extract was measured by dye binding assay as described by Bradford (1976).

Protease activity: Protease activity was determined by the casein digestion assay described by Drapeau *et al.*, (1974). A series of tubes were equilibrated with 2.0 ml of 1% casein at 37°C for 5 minutes. To all the tubes, 100µl of protease extracts was added and mixed well. A reagent blank was also included. Exactly ten minutes after adding sample, reaction was stopped by adding 2.0 ml TCA solution and mixed well. Tubes were then allowed to stand for ten minutes and then reaction solution was filtered to remove the precipitate formed during reaction. The absorbance of filtrate was measured at 280 nm. By this method one unit is that amount of enzyme, which releases acid soluble fragments equivalent to 0.001 A280 per minute at 37°C and pH 7.8. Enzyme activity was expressed on protein basis.

Malondialdehyde (MDA) 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 method of Heath & Packer (1968) with minor modifications as described by Dhindsa *et al.*, (1981) and Zhang & Kirkham (1994). A 0.25 *g* leaf sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10 000 *g* for 5 min. To 1 ml aliquot of the supernatant 4 ml 20% TCA containing 0.5% TBA were added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice-bath. After centrifugation at 10,000 *g* for 10 min the absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted. The MDA content was calculated by using extinction coefficient of $155 mM^{-1}\ cm^{-1}$.

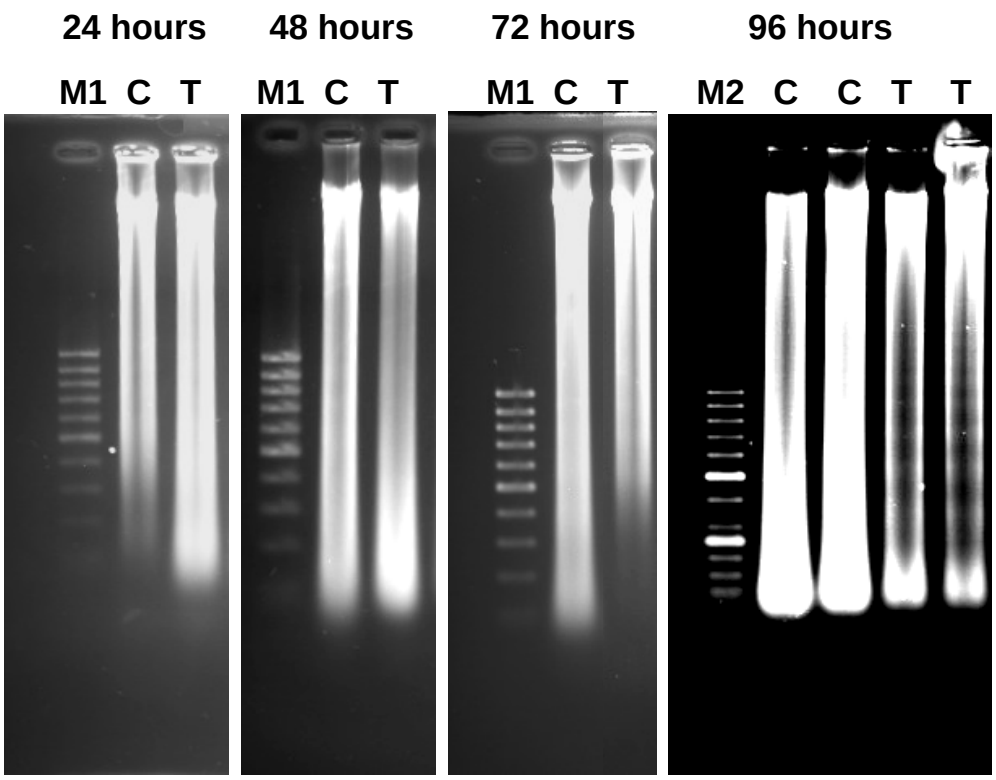
Total phenolic content: A micro colorimetric method as described by Ainsworth & Gillespie (2007) was used for total phenolics assay, which utilizes Folin-Ciocalteu (F-C) reagent. A standard curve was prepared using different concentration of gallic acid and a linear regression equation was calculated. A 0.2 gram of leaf sample was homogenized in 0.8ml ice cold 95% methanol using an ice cold mortar and pestle. The samples were then incubated at room temperature for 48 hours in the dark. The samples were then subjected to centrifugation at 10,000 *g* for 5min at room temperature. The supernatant was removed and used for TPC measurement. A 100µl of supernatant was mixed with 100µl of 10 % (vol/vol) F-C reagent, vortex thoroughly and then 800µl of 700m*M* Na_2CO_3 was added. Samples were then incubated at room temperature for 2 hours. Blank corrected absorbance of samples was measured at 765nm. Phenolic content (gallic acid equivalents) of samples was determined using linear regression equation.

Detection of internucleosomal nDNA fragmentation: To isolate DNA, method described by Hameed *et al*., (2004) with slight modifications was used. For isolation of DNA 0.2g frozen samples were grinded with pre chilled (overnight freezing at -80 ^{o}C) mortar and pestle. Grinded material was immediately transfer into pre warmed extraction buffer (600µl) in labeled 1.5 ml microfuge tubes placed in a water bath at 65 ^{o}C. Samples were incubated at 65 ^{o}C for 10 min with gentle mixing after every 3 min. Microfuge tubes were taken out from water bath and left for 1-2 min at room temperature. To each tube 600µl of chloroform-isoamyl alcohol was add and samples were mixed gently. Samples were centrifuged at 4,000rmp for 5 min. The upper aqueous phase was removed and two volumes of ice-cold 96% ethanol were added to each tube and mix gently for 5 to 10 times to precipitate the DNA. Samples were then centrifuged at 10,000rmp for 10 min to pellet the DNA. The pellet was dissolved in TE buffer. Isolated DNA samples were treated with DNase-free ribonuclease A (50 µg/mL) for 25 min at 37°C. DNA was then precipitated with addition of three volumes of 96% ethanol and samples were centrifuged at 10,000 rpm for 10 min to pellet the DNA which was dissolved in TE buffer. For quantification and purity of DNA, absorbance was measured at 260nm and at 280nm respectively. To check apoptotic internucleosomal nDNA fragmentation, equal amounts of DNA preparations were subjected to electrophoresis in 1.5% agarose gel at constant 100 volts for 3-4 hours. The gel was stained with ethidium bromide and observed under UV transilluminator. Stained gels were photographed using UVI pro-platinum gel documentation system (UVItec UK).

Statistical analysis of data: 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.

Results

Mannose (1%) induced apoptotic internucleosomal nDNA fragmentation after 96 hours of treatment in wheat roots when it was not observed in control (Fig. 1). Protease activity was significantly ($p<0.001$) higher because of mannose treatment at 24 to 72 hours in wheat roots (Fig. 2a). Protease activity was enhanced prior to induction of apoptosis which shows involvement of proteolysis in apoptotic induction process. Maximum protease activity was observed at 72 hours after mannose treatment. Under



C: Control
T: D-Mannose (1 % = 56mM)
M1: SM0243 GeneRuler 100bp DNA ladder (Fermentas) (in bp): 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100.
M2: SM0371 GeneRuler 50bp DNA ladder (Fermentas) (in bp): 1031, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100.

Figure: 1. Electrophoregrams of DNA isolated from roots of etiolated wheat seedlings at different time intervals.

non treated condition protease activity gradually increased with increasing time and was maximum at 96 hours. Protease activity was significantly ($p<0.001$) lower at 96 hours after mannose treatment compared with non treated control. Protein content of roots significantly ($p< 0.001$) increased only at 96 hours after mannose treatment (Fig. 2b). At all other time interval (24 to 72 hours) protein content of roots was decreased due to mannose treatment. Under non treated condition protein content of roots gradually decreased with increasing time and was lowest at 96 hours. With exception of protein level at 96 hours a gradual decreased was also observed under treated condition with increasing time.

Lipid peroxidation in wheat roots increased significantly ($p<0.01$) at 72 hours ($p< 0.001$) after mannose treatment (Fig. 3a). While MDA content of roots remained unchanged at other time intervals. This sharp increase in membrane lipid peroxidation in

wheat roots due to mannose treatment is just prior to apoptotic internucleosomal nDNA fragmentation at 96 hours.

Total phenolic content increased ($p < 0.01$) in wheat roots only at 24 hours while it decreased ($p < 0.01$) at 48 hours after mannose treatment (Fig. 3b). Therefore total phenolic content was down regulated 48 hours before induction of apoptosis. Under non treated condition peak was observed at 48 hours. While in case of mannose treated roots maximum TPC was observed at 24 hours.

Catalase activity in wheat roots increased significantly ($p<0.01$) at 24 48 and 96 hours after mannose treatment (Fig. 4a). An overlapping trend with a peak at 72 hours was observed for catalase activity under non treated and treated conditions however magnitude was higher in later one.

Peroxidase activity in wheat roots after mannose treatment was initially (24 hours) lower than control but slightly higher ($p<0.05$) at 48 hours. At 72 hours it was over two folds ($p<0.001$) in treated roots while remained at the same level in control (Fig. 4b). At 96 hours peroxidase activity sharply declined coming to the level of control roots.

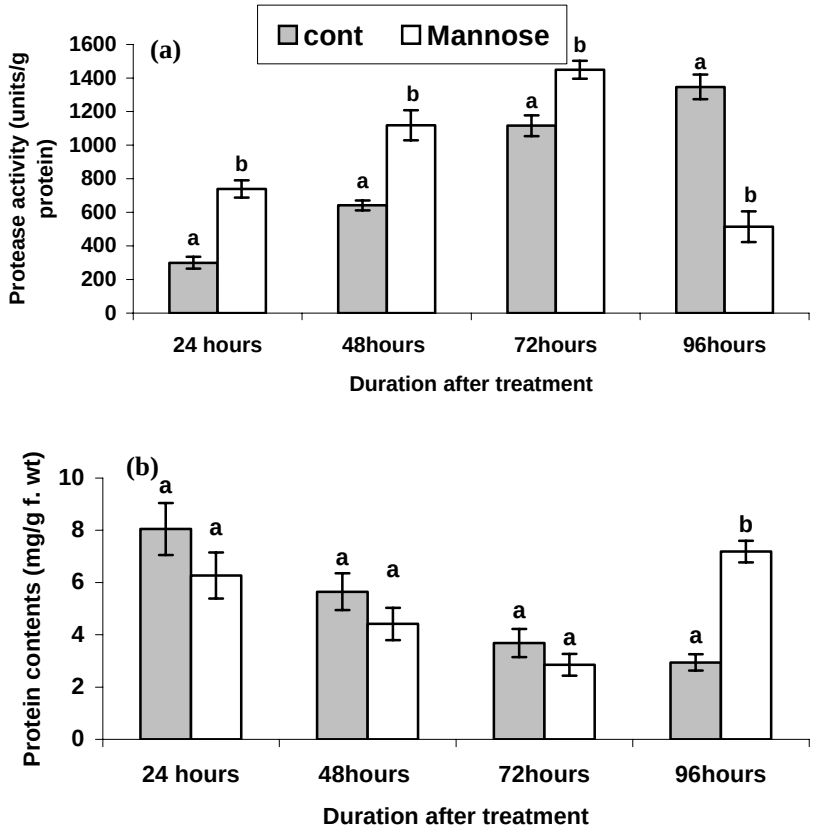


Figure: 2. Protease activity (a) and protein content (b) in wheat (*Triticum aestivum* L.) roots at different time intervals after mannose treatment.

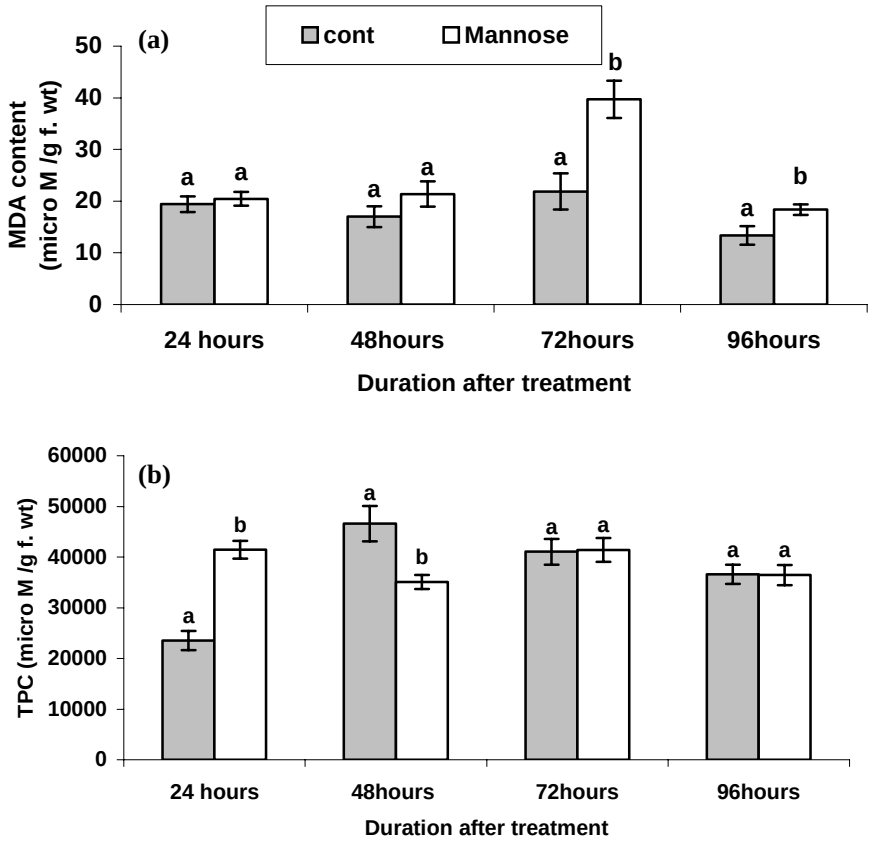


Figure: 3. Malondialdehyde (MDA) (a) and total phenolic content (b) in wheat (*Triticum aestivum* L.) roots at different intervals after mannose treatment.

Superoxide dismutase activity increased significantly ($p<0.001$) at 48 and 72 hours after mannose treatment and maximum activity level was observed at 72 hours after treatment (Fig. 4c). Superoxide dismutase activity was down regulated parallel to induction of apoptosis. At 96 hours after mannose treatment, SOD activity in wheat roots was down regulated and was significantly lower ($P<0.001$) compared with non treated control.

Collectivity antioxidant level was enhanced just before (at 72 hours) initiation of apoptotic process. However antioxidant defense was down regulated and proteases activities and lipid peroxidation were enhanced during induction of apoptosis. These bio molecules thus play a key role in controlling D-mannose induced apoptotic process in wheat roots.

Discussion

Apoptosis is characterized by fragmentation of nuclear DNA by the action of endonucleases. This DNA fragmentation, which is an important event in cell death

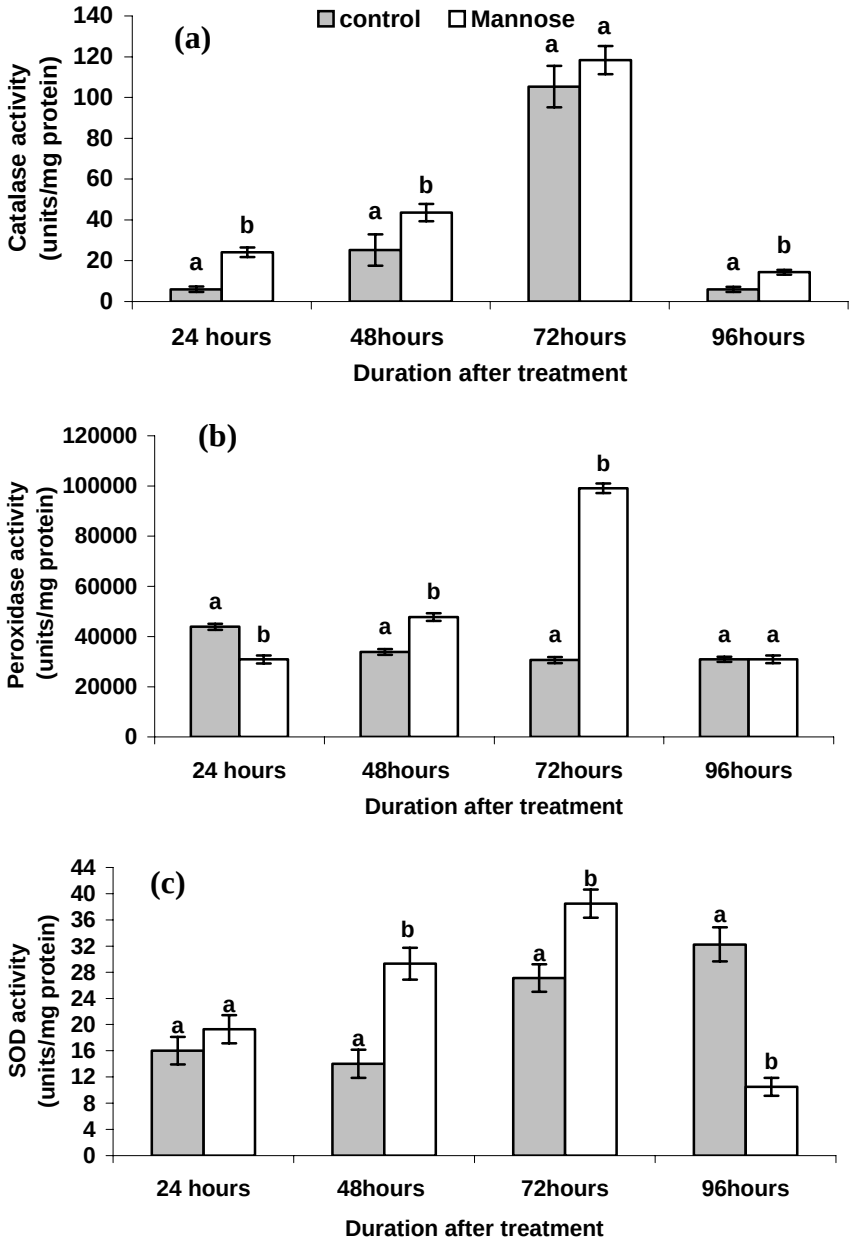


Figure: 4. Activities of catalase (a), peroxidase (b) and superoxide dismutase (c) in wheat (*Triticum aestivum* L.) roots at different intervals after mannose treatment.

pathway, has been used as a hallmark of apoptosis (Zamyatnina *et al*., 2002). Cell under going active apoptosis yields highly fragmented DNA, which appear as a smear, or ladder in electrophoresis gels. In present study D-mannose (1%) induced apoptotic internucleosomal nDNA fragmentation in wheat roots after 96 hours of treatment while it was not observed in control. This is first report providing evidence for apoptotic effect of D-mannose in wheat roots. Previously D-mannose has been shown to induce apoptosis in arabidopsis roots and maize (*Zea mays*) suspension-cultured cells which was also assessed by internucleosomal fragmentation caused by endonuclease (Stein & Hansen, 1999).

Several possible routes by which D-mannose could induce apoptosis have been reported including interference with glucose utilization and phosphate availability (Goldsworthy & Street, 1965) and compromising the cell's ability to detoxify reactive oxygen species (Stein & Hansen, 1999) by lowering ATP required for the regeneration of reduced pyridine nucleotides and glutathione in erythrocytes (Lachant & Zerez, 1988). Enzymatic antioxidant defense which detoxify the reactive oxygen species (oxidants) was also down regulated during induction of apoptosis in present study. Data presented thus provide evidence that mannose also inhibits defence against oxidants in plant system. No such prior evidence is available. It also point towards the possibility that oxidative stress through enhanced production of reactive oxygen species may be a route by which D-mannose induced apoptotic effects.

Protein breakdown and recycling, which depend on the levels of proteolytic enzymes, are an essential part of the plant response to environmental stress (Hieng *et al.*, 2004, Hamid *et al.*, 2008; Siddiqi & Ashraf, 2008). Mannose treatment decreased the protein content of roots at most time intervals. This decrease in protein content overlaps with increased protease activity. Previously increase in proteases along with decrease in protein content and increase in free amino acids content has been reported in *B. parviflora* (Parida *et al.*, 2002, 2004) and radish (Muthukumarasamy *et al.* 2000) under salt stress. Results being reported in the present study supported by published data suggested that decreased protein content relates with increased protease activity. Proteases are actually involved in remove of abnormal, misfolded proteins, and protein rebuilt in response to different external stimuli (Grudkowska & Zagdanska, 2004).

Protease activity enhanced prior to induction of apoptosis which shows involvement of proteolysis in mannose induced apoptotic process. Proteolytic enzymes seem to take part in each of three stages of apoptosis starting from the moment of the appearance of the apoptosis induction signal up to strong destruction of structural proteins and enzymes that maintain the cell homeostasis. Further enzyme inhibitory analysis has provided evidences that the cysteine and serine proteases play a main role in apoptosis in wheat coleoptiles (Fedoreyeva *et al.*, 2003). Proteolytic enzymes are known to be associated with developmentally programmed cell death during organ senescence and tracheary element differentiation (Beers *et al.*, 2000). Some evidences also links proteinases with some types of pathogen and stress induced cell suicide (Beers *et al.*, 2000). The plant proteinases may act as mediators of signal transduction and as effectors of programmed cell death.

Breakdown of membrane due to lipid peroxidation is among one of the factors underlying the increased proteolytic activities during senescence which is a type of programmed cell death (Storey & Beevers, 1978). Similarly in present study along with increased proteolytic activity, peroxidation of membrane lipids also increased as a result

of mannose treatment. This sharp increase in membrane lipid peroxidation in wheat roots due to mannose treatment is just prior to apoptotic internucleosomal nDNA fragmentation at 96 hours Therefore it seems that breakdown of membrane as a consequence of mannose treatment is also one of the factors responsible for increased proteolytic activity. Enhanced protease activity might be for proteolysis of membrane bound proteins released due to breakdown of membrane. The ability of reactive oxygen species (ROS), to initiate lipid peroxidation has been demonstrated (Kellogg & Fridovich, 1975; Brennan & Frenkel, 1977). Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Smirnoff, 1993; Alscher *et al*., 1997; McCord, 2000). Enhanced lipid peroxidation after mannose treatment thus provides second evidence for involvement of reactive oxygen species in execution of mannose induced apoptosis.

Plants detoxify ROS by up-regulating antioxidative enzymes, like superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). A main role is attributed to SOD in catalysing the dismutation of superoxide anions to dioxgyen and hydrogen peroxide (H_2O_2) (Raza *et al*., 2007). The increased production of H_2O_2 is subsequently counteracted by peroxidase or catalase (Garratt *et al*., 2002). Mainly antioxidants determine the abundance of ROS in the tissue, and thereby control the level of lipid peroxidation. That's way in present study, lipid peroxidation was not increased during initial time period when level of most of antioxidants i.e. catalase, peroxidase and phenolics was higher and system was efficiently removing excessive ROS. However during later stage (at 96 hours) when antioxidant defence was down regulated and system was unable to remove excessive ROS, degradation of membrane lipids and nDNA was observed. Inability to maintain antioxidant activities by root tissue most probably resulted into uncontrolled ROS and therefore enhanced membrane lipid peroxidation and ultimately induction of apoptosis.

The model system used in the present study thus presents the potential to study the induction and regulation of apoptosis in plants. It can be concluded that mannose induced apoptotic internucleosomal nDNA fragmentation in wheat roots as a consequence of reduced antioxidant activities, increased protease activity and extensive peroxidation of lipids. Indirect evidences points towards central role of excessive ROS during induction of mannose induced apoptosis however direct evidence is still considered necessary.

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