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EFFECT OF POLYAMINES ON SEEDLINGS OF TWO MANGO (*MANGIFERA INDICA* L.) ROOTSTOCKS UNDER SALT STRESS

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SUMMARY

A study was conducted to investigate the effects of polyamines (putrescine and spermidine) on photosynthetic pigments, relative water content (RWC), membrane injury (MI), proline content and activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in 1-year-old seedlings of mango rootstock Kurukkan and non-descript mango under NaCl stress. Results indicated that polyamines mitigated the salinity stress and reduced membrane injury of mango seedlings by 57% (Spd) and 27% (Put) 60 days after salt treatment over non-treated salinised plants. Polyamines increased the endogenous proline content, and salinised plants treated with polyamines had 35% (Spd) and 21% (Put) higher proline content than salinised plants without polyamines treatment. Higher antioxidant enzyme activity was also observed in salinised plants treated by polyamines than salinised plants without polyamine treatments. The effect of spermidine was greater than putrescine in terms of activity of antioxidant enzymes in mango rootstocks. In comparison to salinised plants without polyamines treatment, salinised plants treated with spermidine had higher SOD (12.0%) and CAT (11.3%) activities 60 days after salt treatment. Our results suggest that modulating effect of polyamines on mango rootstock plants under salt stress may be attributed to increased photosynthetic pigments, relative water content, proline and antioxidant enzymes activity and by reducing membrane injury in mango rootstocks.

Key words: **Mango, putrescine, rootstock, salinity, spermidine.**

INTRODUCTION

Mango is considered as the most popular tropical fruit of the world and has been rightly described as 'King of fruits' in India, owing to its historical and commercial importance, delicious taste, capitative flavour and attractive aroma. Mango is now grown commercially in more than 90 countries, but nowhere it is more extensively cultivated than in India, where it occupies 2.3 mha area with an annual production of 15.0 million tons (NHB 2010). It is a matter of concern that the productivity of mango orchards in India is low (6.5 t ha^{-1}). Several factors are responsible for the poor productivity of mango orchards, of which salinity of soil and water are most important. The presence of salts in the irrigation water, or in the soil, alters the nutritional balance of plants that can inhibit the growth of plants. Salt stress, in addition to osmotic stress effects and ion toxicity, is also manifested as oxidative stress (Hernandez *et al*. 1993, Gomez *et al*. 1999). Salt stress results in a wide variety of physiological and biochemical changes in plants. Among these, accumulation of low-molecular-

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weight solutes such as proline, commonly referred to as compatible solutes, and changes in free polyamine levels are significant events. The physiological significance of these responses in higher plants is a matter of controversy because direct evidence for the part played by polyamines during acclimation to stress conditions remains tenuous (Aziz *et al.* 1999).

Polyamines (PAs), such as putrescine (Put), spermidine (Spd), and spermine (Spm), are ubiquitous in plant species, and have been reported to regulate synthesis of proteins and RNA (Tabor and Tabor 1985), cell division and organ growth (Galston and Kaur-Sawhney 1990), and plant senescence (Altman 1982). Possible role of PAs have been suggested in plant defense responses to various types of oxidative stresses, e.g., UV radiation (Kramer *et al.* 1991), salt stress (Aziz *et al.* 1997), acid rain (Velikova *et al.* 2000), and heavy metals (Groppa *et al.* 2001). All of these stresses, which inhibit normal plant growth, lead to the cellular accumulation of reactive oxygen species (ROS), such as superoxide radicals and H_2O_2. Drolet *et al.* (1986) reported that PAs can function directly as ROS scavengers. However, Bors *et al.* (1989) have discounted their scavenging capability, while confirming the protective influence of these molecules against ozone-induced oxidative damage. Velikova *et al.* (2000) have also reported that PAs may prepare the cell to counteract stress by forming a higher potential of cellular antioxidant systems. Thus, the exact mechanism of PAs actions for enhancing the defense response in plants still remains unclear.

Keeping in view the above facts, the present investigation was conducted with the objectives to elucidate the role of polyamines in ameliorating growth and associated metabolic activities of mango seedlings subjected to salt stress.

MATERIALS AND METHODS

Experimental materials: The present study was conducted with seedlings of two mango rootstocks *viz.*, polyembryonic mango *cv.* Kurukan and monoembryonic mango plants (non-descript). Fruits were harvested and stones were sown in July 2007 after extraction from the pulp. Germinated seedlings were allowed to grow until 30 June 2008. In July 2008, nucellar plants of Kurukan and zygotic plants of non-descript mango were transferred to 20 kg capacity brick red colour plastic pots (height 35 cm and diameter 35 cm), filled with orchard soil (sandy loam, typic haplustep) having pH 7.17, and EC 0.28 dS m^{-1}, CEC 10.65 cmol kg^{-1} soil, and an organic carbon content of 4.3 g kg^{-1} soil. One kg well rotten F.Y.M. was added to each pot at the time of transplanting of seedlings. Plants were irrigated at weekly interval and fertilized with 30 g fertilizer mixture consisting of urea, single super phosphate and potassium sulphate (1:1:1) and were allowed to grow for 30 days.

Experimental details: Salt treatment was given to the plants twice at 7 days interval after 30 days of transplanting when plants were properly established in the pots. One litre solution of 150 mM NaCl was poured in pots twice at 7 days interval. Each treatment was replicated ten times. Plants grown in pots were again treated twice with 0.6 mM putrescine and 0.7 mM spermidine at 7 days interval. The electrical conductivity (EC) of the soil was tested at regular interval (10 days). The mean soil EC values recorded at end of the experiment were: 0.41 dS m^{-1} (non-stressed control + no-polyamine); 0.41 dS m^{-1} (non-stressed + 0.6 mM putrescine); 0.42 dS m^{-1} (non-stressed + 0.7 mM spermidine); 2.68 dS m^{-1} (salt-stressed + no-polyamine); 2.70 dS m^{-1} (salt-stressed + 0.6 mM putrescine) and 2.66 dS m^{-1} (salt-stressed + 0.7 mM spermidine). During the experiments, the pots were manually irrigated with water (EC 0.22 to 0.31 dSm^{-1}) at 3 days interval considering the moisture loss measured by direct weighing of pots.

Number of leaves per plant were counted from each seedling from the date of salt treatment at 20 days interval. Defoliation was calculated for each seedling at an interval of 20, 40 and 60 days after salt treatment and expressed in percentage.

The relative water content (RWC) of recently matured leaves was determined using the method of Barrs and Weatherley (1962). Leaves were collected and 8 mm-diameter leaf discs were cut. The fresh weight of these discs was measured, and they were then floated on distilled water for 4-6 h in petri plates. The discs were then surface dried by placing them between two sheets of Whatman No. 1 filter paper. The saturated weight

(i.e., turgid weight) of discs was recorded, and thereafter dried to constant weight in an oven at 70 ºC for 48 h and their dry weights were recorded. The relative water content was estimated using the formula, RWC (%) = [(Fresh weight – Dry weight) / (Turgid weight – Dry weight)] x 100

The membrane injury index (MII) of three leaves per replication was examined using the method of Deshmukh *et al.* (1991). Fresh leaf material (0.5 g) was weighed and placed in a test-tube containing 10 ml double-distilled water. The tubes were incubated at 45 ºC for 30 min in a water bath. The EC of the solution was then measured using a conductivity meter (Model-146; Systronics India Ltd., Mumbai, India). The test-tubes were then placed in a boiling water bath for 10 min, cooled to room temperature, and their EC values were measured once again. The method was standardised by repeated observations for uniform results. MII was determined by the formula, MII = (EC at 45 ºC/ EC at 100 ºC) x 100.

The chlorophyll contents (chlorophyll *a, b,* and total chlorophyll) of leaves were analysed following the DMSO method of Barnes *et al.* (1992). The absorbance of samples was then read at 645 and 663 nm, using pure DMSO as the blank in a UV-VIS spectrophotometer (Model 5704; Electronic Corp. of India Ltd., Hyderabad, India).

Proline content was estimated using a rapid colorimetric method of Bates *et al.* (1973). Fresh leaf (0.5 g) samples were homogenized in a pre-chilled mortar and pestle with 5 ml of 3% sulfosalicylic acid. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant was diluted to 10 ml with double-distilled water. The 0.1 ml of the diluted extract was placed in a test tube and further diluted to 1 ml followed by addition of 5 ml each of acid ninhydrin reagent and glacial acetic acid. The tubes were heated for 1 h at 100 ºC in a hot water bath. The reaction was terminated by keeping the test tubes in an ice bath followed by addition of 4 ml of toluene and stirred vigorously for 20-30 s. The chromophore in toluene layer (light pink) was aspirated from the aqueous phase and warmed to room temperature, and then the absorbance was read at 520 nm on a UV–VIS spectrophotometer (UV–VIS 5704SS) by using pure toluene as a blank. The proline concentration in the samples was determined from a standard curve prepared by using analytical grade proline (SRL Chemical Company, Mumbai, India).

Leaves were excised, rapidly weighed (1.0 g fresh weight) and ground in pre-cooled mortar and pestle with 10 ml 50mM phosphate buffer (pH 7.0). The homogenates were centrifuged at 20,000×g for 30 min at 4 ºC. The supernatant filtered through two layers of cheese-cloth were used for the assays of enzymatic activities.

The SOD activity was determined according to the method of Fridovich (1975). One enzyme unit of SOD activity was defined as the amount of enzyme required to cause 50% reduction in the absorbance of formazone formed in the absence of enzyme. The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.0), 200 mM methionine, 1.125 mM NBT, 1.5 mM EDTA, 75 µM riboflavin, and10–40 µL of enzyme extract. Riboflavin was added as the last component. The tubes were shaken and placed 30 cm below a light bank consisting of two 15-W fluorescent tubes. The reaction was started by switching on the light and allowed to run for 10 min, and switching the light off stopped the reaction. The tubes were then immediately covered with black cloth and the absorbance was measured at 560 nm (UV–vis spectrophotometer, 5704, Electronic Corporation India Ltd.). The non-irradiated reaction mixture had zero absorbance (log A560), which was plotted as a function of the volume of the enzyme extract in the reaction mixture. The volume of the enzyme extract producing 50% inhibition of the reaction was read from the resultant graph.

The catalase activity in leaves was determined by employing the method suggested by Luck (1975). Catalase activity was assayed by estimating the residual H_2O_2 by oxidation with KMnO4 titrimetrically. The reaction mixture consisted of 3 ml of phosphate buffer (0.1 M, pH 7.0), 30 µL of H_2O_2 (5 mM) and 1 ml of enzyme extract was then incubated in a test tube at 20^0C for 1 min. The reaction was stopped by adding 10 ml of 0.35 M H_2SO_4 and the residual H_2O_2 estimated by titrating the reaction mixture against 0.01M $KMnO_4$. The end-point for the titration was a faint purple colour,

which persisted for at least 15 s. A blank was prepared by adding enzyme extract to an acidified solution of reaction mixture at zero time. The enzyme activity was expressed as μmol of H_2O_2 10 min^{-1} g^{-1} of fresh weight of leaves.

The peroxidase activity in leaves was estimated using the method of Thomas *et al*. (1981). Peroxidase was assayed using guaiacol as the substrate. The reaction mixture consisted of 3 ml of phosphate buffer (0.1 M, pH 7.0), 30 μL of H_2O_2 (20 mM), 50 μL of enzyme extract and 50 μL of guaiacol (20 mM). The reaction mixture was incubated in a cuvette for 10 min at room temperature. The optical density was measured at 436 nm. The enzyme activity was expressed as number of absorbance units g^{-1} fresh weight of leaves.

The experiment was laid out in complete randomized design (CRD). The data was analysed by using analysis of variance (ANOVA) as suggested by Gomez and Gomez (1984).

RESULTS AND DISCUSSIONS

Effect of NaCl stress on defoliation was significant ($P \leq 0.05$) and salt-stressed plants showed very high degree of defoliation than unstressed plants. The tip and marginal necrosis of leaves was more evident in salt stressed plants of mango. Regardless of polyamine treatments, defoliation was more pronounced in non-descript mango plants (23.61%) compared to Kurukan plants (19.39%). This indicates comparatively susceptible nature of non-descript mango plants against salt stress compared to Kurukkan plants. This finding is in conformity with the observations of Dubey *et al*. (2007) and Srivastav *et al*. (2009), who reported Kurukan as moderately salt tolerant mango rootstock. Polyamines treated plants under salt stress have a lower proportion of leaves showing marginal and tip necrosis, and there was gain in number of leaves in due course of plant development. Leaf fall was significantly reduced by application of polyamines in both mango rootstocks and the effect of spermidine (2.18 folds reduction) was more than putrescine (1.54 folds reduction). The alleviating effect of polyamines may be attributed to the fact that polyamine application consistently reduced the Na^+ and Cl^- contents in the leaves under salinity. Moreover, under saline condition polyamine application caused increase in chlorophyll contents, proline and antioxidant enzymes activity in plants and allowed plant to develop well.

Relative water content was significantly affected by salt stress, rootstock and polyamines. Maximum RWC was observed in non-salt-stressed Kurukan plants treated with spermidine on 20th day (88.9%) and 40th day (88.9%). On 60th day, RWC was higher in non-salt-stressed Kurukan plants without polyamine treatment (89.8%). The minimum RWC was observed on 20th day in salt-stressed Kurukan plants without polyamine treatment. However, on 40th and 60th day RWC was lowest in salt-stressed non-descript mango plants without polyamine treatment (66.3 and 62.4%, respectively). It is evident from the results presented in Table 1 that in non-salt-stressed condition polyamine had non-significant effect on RWC in both the rootstocks. In contrast application of polyamines in salt-stressed conditions resulted in significant increase in RWC in both the rootstocks. Increase in RWC under salt-stressed condition was significantly greater due to spermidine treatment compared to putrescine treatment over salt-stressed controls in both the rootstocks.

Salt stress significantly increased MII at all the dates compared to non-stressed mango plants. It was also interesting to note that with increase in the age of plants, the MII increased significantly in salt stressed plants compared to non stressed plants. On 20th, 40th and 60th day, MII in salt stressed plants was 122.24, 243.64 and 280.14%, respectively higher compared to non-stressed plants. It shows that continuous damage occurred under salt stress due to physiological changes. The polyamine treatment decreased the extent of membrane damage in mango plants, and spermidine application was more beneficial than putrescine at all the dates (Table 1). Stress induced production of secondary metabolites, such as polyamines, have been reported to act as protective mechanism against stress damage (Zhu 2002; Wahid *et al*. 2007). Polyamines interaction with membrane phospholipids has been reported to provide membrane stability under stress conditions (Roberts *et al*. 1986). Since PAs can act as free radical scavengers, these may also protect the membranes from oxidative damages (Besford *et al*. 1993). Loss of integrity of biological membranes, principally due to the oxidative damage, is

Table 1. Effect of rootstock, salinity and polyamines on chlorophyll a, b and total chlorophyll content, relative water content, membrane injury and proline in seedlings of two mango rootstocks.

Treatment	Chlorophyll 'a' mg g^{-1} fr. wt.			Chlorophyll 'b' mg g^{-1} fr. wt.			Total chlorophyll mg g^{-1} fr. wt.			RWC (%)			MII		
DAST	20	40	60	20	40	60	20	40	60	20	40	60	20	40	60
R1S0P0	3.12	3.19	3.22	0.83	0.71	0.68	3.97	3.92	3.90	86.8	87.7	89.8	5.5	8.3	10.2
R1S0P1	2.88	3.02	3.01	0.77	0.68	0.66	3.61	3.71	3.68	89.4	86.9	88.4	7.4	10.6	11.1
R1S0P2	2.97	3.12	3.23	0.73	0.70	0.70	3.75	3.81	3.93	86.5	88.9	89.4	5.8	10.5	11.5
R1S1P0	1.97	1.46	1.07	0.64	0.51	0.41	2.65	1.95	1.43	77.1	69.1	66.3	20.4	41.1	54.1
R1S1P1	2.17	2.08	1.65	0.71	0.62	0.57	2.89	2.72	2.23	80.0	73.3	71.3	10.6	25.7	35.9
R1S1P2	2.56	2.33	2.12	0.76	0.64	0.61	3.31	2.94	2.72	81.3	79.7	81.4	8.6	18.1	21.9
R2S0P0	2.64	2.96	3.00	0.72	0.86	0.78	3.34	3.80	3.77	86.4	85.9	88.4	7.1	9.3	12.3
R2S0P1	2.54	2.57	2.87	0.63	0.75	0.72	3.19	3.35	3.61	84.7	87.9	89.4	8.4	10.4	10.9
R2S0P2	2.70	2.74	2.79	0.63	0.72	0.75	3.31	3.44	3.55	88.9	87.3	88.8	7.6	8.9	12.1
R2S1P0	2.07	1.27	0.87	0.56	0.37	0.21	2.64	1.65	1.06	75.8	61.3	62.4	24.5	54.9	65.4
R2S1P1	2.22	1.67	1.55	0.61	0.49	0.38	2.85	2.16	1.89	77.9	75.9	70.3	16.4	38.2	51.3
R2S1P2	2.36	2.43	2.15	0.64	0.64	0.52	3.04	3.06	2.66	79.2	83.2	78.6	12.3	21.3	29.6
LSD *(p ≤0.05)*															
Rootstock (R)	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.03	0.65	NS	0.76	0.67	0.86	0.88
Salinity (S)	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.03	0.65	1.01	0.76	0.67	0.86	0.88
Polyamines (P)	0.02	0.02	0.01	NS	0.01	0.10	0.04	0.04	0.04	0.92	1.41	1.07	0.95	1.22	1.24
R x S	0.01	0.01	0.01	NS	0.01	0.02	0.04	NS	0.04	NS	NS	0.93	0.83	1.06	1.08
R x P	0.02	0.02	0.01	0.02	0.02	0.02	0.06	0.05	NS	1.12	1.72	1.31	NS	1.49	1.52
S x P	0.02	0.02	0.01	0.02	0.02	0.02	0.06	0.05	0.05	1.12	1.72	1.31	1.17	1.49	1.52
R x S x P	0.03	0.03	0.02	0.02	0.02	0.02	0.08	0.06	0.06	1.58	2.43	1.85	N.S	2.11	2.15

R1, Kurukkan; R2, Non-descript seedlings; S0, Non-salt-stressed (Control); S1, Salt-stressed; P0, No-polyamine (control); P1, Putrescine (0.6 mM); P2, Spermidine (0.7 mM); *DAST, days after salt treatment

one of the effect of abiotic stresses (Liu *et al*. 2000). Polyamines are basic molecules, which are positively charged at physiological pH. They are shown to bind strongly with negatively charged nucleic acids and acidic phospholipids on plasma membrane. These ionic interactions are important to plants under salt stress in preventing degradation of biological macromolecules and alleviating membrane damage (Basra *et al*. 1994). Exogenous PAs could protect the integrity of plasma membrane and tonoplast under salinity and regulate the absorption and allocation of ions in plant cells. Several lines of evidences have also shown that the stimulatory effect of exogenously applied polyamines may be related to their multifaceted nature, which includes working as an antioxidant, a free radical scavenger and a membrane stabilizer (Velikova *et al*. 2000).

The NaCl treatment with or without polyamines decreased chlorophyll 'a', 'b' and total chlorophyll in comparison to non-salt-stressed control. However, application of polyamines under salt stress increased chlorophyll contents in both the rootstocks. Chlorophyll contents in salt-stressed plants were reduced gradually with age. Furthermore, the effect of spermidine was more pronounced than putrescine in increasing the chlorophyll 'a', 'b' and total chlorophyll contents in both the rootstocks under salt stress (Table 1). Result obtained in present investigation clearly suggests the role

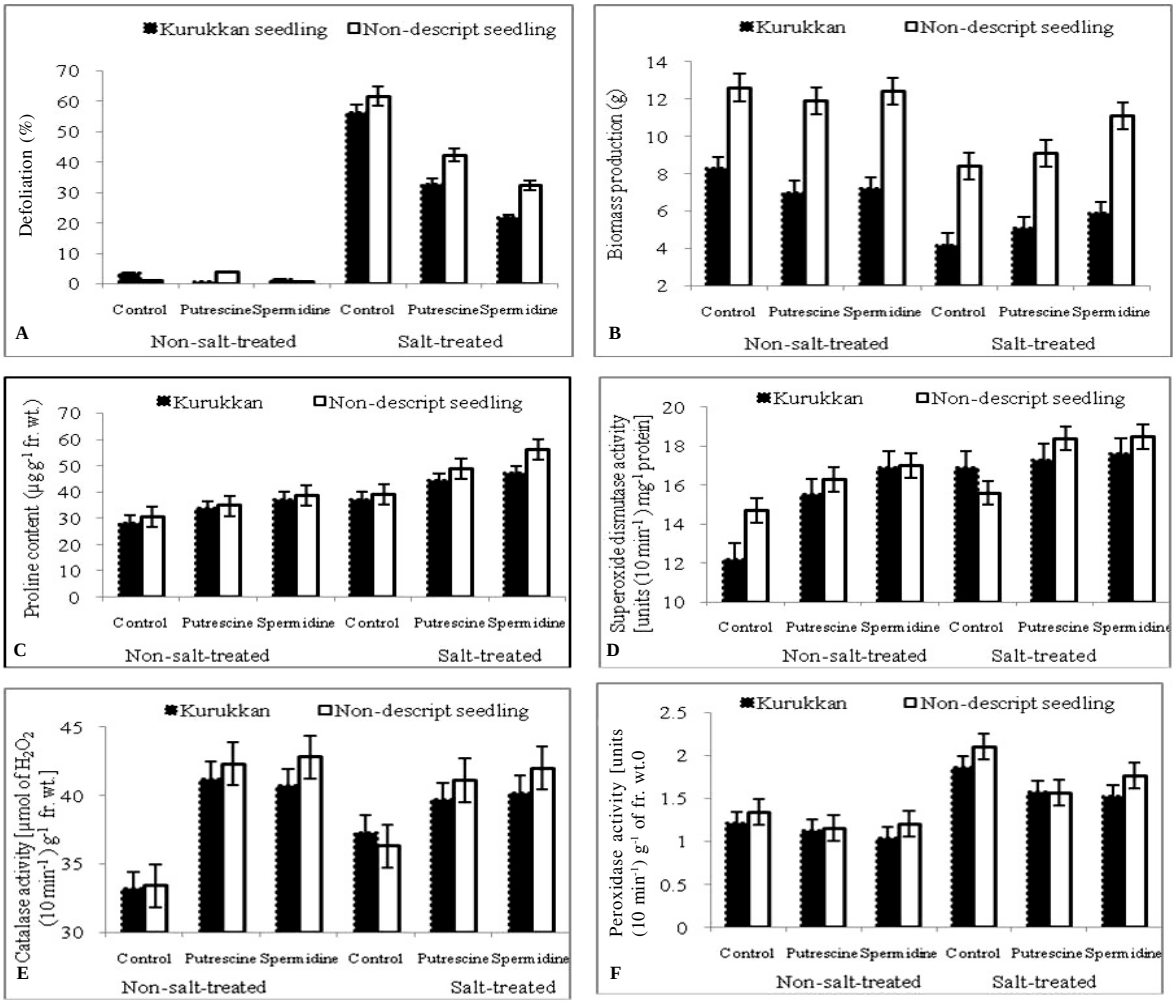

Fig. 1. Effect of polyamine (Put and Spd) and salt stress on defoliation (A), biomass production (B), proline content (C), activities of super oxide dismutase (D), catalase (E) and peroxidase (F) in seedlings of two mango rootstocks, Kurukkan and non-descript mango seedlings. A significant interaction among genotype, salinity and polyamines was found (P≤0.05). Vertical bars indicates ± SE.

of polyamines in ameliorating the inhibitory effects of NaCl on chlorophyll contents in leaves. Photosynthesis, one of the most important metabolic pathways in plants, is a target of salt stress. Chlorosis of leaves is the first visual symptoms of stress leading to senescence (Fletcher and Hofstra 1985), and is associated with a concomitant decline in concentration of photosynthetic pigments (Fletcher and Hofstra 1985, Pinhero and Fletcher 1994). Maria *et al.* (2000) examined the damage to the photosynthetic apparatus under paraquat stress. The pigment content decreased by 30% in stressed discs after 20 h of treatment. However, chlorophyll loss was completely prevented by spermidine pre-treatment and spermine restored chlorophyll up to 90% of the control value but putrescine was not effective. Our results are also in agreement with the observations made by Zeid (2004), Kim and Jin (2006) and Sotiropoulos (2007).

Both salinity and polyamine treatment significantly increased proline content in the two mango rootstocks. Irrespective of rootstock and polyamine treatments, salt stress increased proline content (34.16%) compared to non stressed plants. Proline content was increased by 19.35% and 32.46% by the application of putrescine and spermidine, respectively compared to non-polyamine treated plants. Proline accumulation in osmotically stressed tissues is considered to be involved in osmotic adjustment (Hare and Cress 1997) and also to be a nitrogen source for recovery from stress (Trotel *et al.* 1996). Moreover, proline is considered to be part of a general adaptive response to other adverse environmental conditions, such as low temperature, heavy metals or nutrient deficiency (Chiang and Dandekar 1995, Delauney and Verma 1993).

Salt stressed Kurukkan plants showed 38.52% more superoxide dismutase (SOD) activity compared to non stressed plants. However, salt-stressed non-descript mango plants showed only 6.12% increase in SOD activity over control. Polyamine treatments significantly increased the SOD activity under salt stress in both the rootstocks. Application of spermidine resulted in 44.26 and 25.85% higher SOD activity in Kurukan and non-descript mango plants over unstressed control. Results suggest that polyamines have a role in enhancing the activity of SOD activity in leaves of both the mango rootstocks under salt stress. Similarly, polyamines treatment increased the CAT activity both under salt stressed and unstressed conditions in both the rootstocks.

Irrespective of mango rootstocks and polyamine treatments, salt-stressed plants showed 47.45% more peroxidase (POD) activity as compared to non-stressed plants. The effect of polyamine treatments were also significant and application of spermidine resulted in decrease in POD activity compared to control plants. The interaction effect of rootstock, salt and polyamines was statistically significant ($P \leq 0.05$). The highest POD activity was observed in salt-stressed non-descript mango plants without polyamine and lowest activity was observed in non-stressed Kurukan plants without polyamine.

Tang and Newton (2005) have also reported that polyamines ameliorated salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreased lipid peroxidation. Present results further corroborated the results reported by Baranenko (2006) and Kartashov *et al.* (2008). Catalase enzyme is an important antioxidant system that catabolises hydrogen peroxide (Larson *et al.* 1988, Smironoff 1993). Our results showed positive effects of spermidine and putrescine on CAT activities both in unstressed and salt stressed plants. Similar results were reported by Liu *et al.* (2007), who have reported that exogenous application of polyamines increased the catalase activity, along with the accruement of proline, an osmo-protectant. Peroxidase catalyses hydrogen peroxide dependent oxidation of cell wall consituents and are involved in lignin synthesis (Badiani *et al.* 1990, Dwivedi *et al.* 1979). Findings of the present investigation pertaining to POD activity in salt-stressed and polyamine-treated mango plants are in accordance with the findings of Ozturk and Demir (2003).

CONCLUSIONS

Thus, it can be concluded from the present investigation that Kurukan and non-descript mango seedlings showed differential response under salt-stressed and unstressed conditions with or without polyamine. Under salt-stressed condition non-descript

mango seedlings showed comparatively susceptible nature than Kurukan. Polyamines played vital role in alleviating the NaCl stress in mango rootstocks. Application of spermidine and putrescine alleviated salt stress induced negative effects to varying degree in both the rootstocks. The effect of spermidine was more pronounced than putrescine modulating effect of polyamines on mango plants under salt stress may be attributed to increased photosynthetic pigments, relative water content, proline accumulation and enhanced activities of antioxidant enzymes and by reducing membrane injury in mango plants.

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