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Effect of Putrescine and Paclobutrazol on Growth, Physiochemical Parameters, and Nutrient Acquisition of Salt-sensitive Citrus Rootstock *Karna khatta* (*Citrus karna* Raf.) under NaCl Stress

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Abstract Salinity is a serious problem in arid and semi-arid areas and citrus trees are classified as salt-sensitive. Because putrescine (Put) and paclobutrazol (PBZ) are known to act as plant protectants under environmental stresses, we examined the effect of Put and PBZ on the physiochemical parameters of the salt-susceptible citrus rootstock *Karna khatta* under NaCl stress. PBZ was applied at 0, 250, and 500 mg L^{-1} as a soil drench 1 week prior to salinization. A computed amount of NaCl salt to develop soil salinity of 3 dS m^{-1} (3 g NaCl kg^{-1} soil) and foliar spray of Put at 0 or 50 mg L^{-1} were applied. The electrical conductivity (EC) of the garden soil (0.35 dS m^{-1}) was used as control. Application of PBZ and/or Put reduced the membrane injury index and increased relative water content, photosynthetic rate, and pigments content under saline conditions compared to what occurred in plants exposed to NaCl in the absence of PBZ or Put. Application of PBZ or Put alone or in combination also improved the activities of SOD and peroxidase and proline content under saline conditions. Application of PBZ and/or Put also increased K^+ and reduced Na^+ and Cl^- concentrations in leaf tissues. It is proposed that PBZ and/or Put could improve the tolerance of salt-susceptible *Karna khatta* by regulating absorption and accumulation of ions and improving antioxidant enzyme activities.

Keywords Citrus · *Karna khatta* · PBZ · Physiochemical parameters · Putrescine · Nutrient acquisition · Salinity

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Introduction

Citrus species are classified as salt-sensitive and are grown preferentially in semiarid areas where irrigation is required to produce a maximum yield. In these areas, soil and water often contain excessive concentrations of soluble salts. Soil salinity is the most important factor responsible for poor productivity of citrus trees. Salinization is one of the serious problems confronting sustainable agriculture in irrigated production systems in arid and semiarid regions (Marschner 1995; Ravindran and others 2007). Salt induces various biochemical and physiological responses in plants and affects almost all metabolic processes (Nemoto and Sasakuma 2002). One of the biochemical changes that occurs when plants are subjected to salt stress is the production of reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). ROS can have detrimental effects on normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Mittler 2002). Most halophytes react to environmental stresses with an effective ROS-scavenging system involving antioxidant enzymes

such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (Greenway and Munns 1980; O'Neill 1983). Therefore, technologies to improve antioxidant enzyme activities and regulate translocation and accumulation of ions under salt stress conditions in citrus would be beneficial.

Polyamines (PAs) such as spermidine, spermine, and their diamine obligate precursor, putrescine, are small aliphatic amines that are ubiquitous in all plant cells. Polyamines interact with negatively charged macromolecules such as DNA, RNA, proteins, and phospholipids in such a way that they are involved in the regulation of the physical and chemical properties of membrane structure and functions of nucleic acids and modulation of enzyme activities (Galston and Sawhney 1990). In the last few years, there has been a growing interest in the study of polyamine participation in the defense reaction of plants against several environmental stresses (Kumar and others 1997; Bouchereau and others 1999; Kasukabe and others 2004). Putrescine ameliorated growth in stressed seedlings, inhibited Na^+ and Cl uptake, accelerated the accumulation of K^+, Ca^{2+}, Mg^{2+}, and proline in the leaves, stabilized cell membranes, influenced protein and nucleic acid synthesis of salt-stressed plants (Krishnamurthy 1991; Singh and others 2000; Tang and Newton 2005), and inhibited stress-induced senescence (Altman and Bacharach 1981).

Triazoles have been called plant multiprotectants because of their ability to induce tolerance in plants to environmental and chemical stresses (Fletcher and Hofstra 1988). Paclobutrazol (PBZ) [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1-PBZ)] is a growth retardant and could inhibit gibberellic acid (GA) biosynthesis, increase cytokinin content, increase abscisic acid (ABA) levels, and decrease ethylene content (Mackay and others 1990). Earlier research indicated that PBZ application reduced the effects of salt stress on guava (Elaidy and others 1992), grape (Salama and others 1992), peach (Abou El-Khashab and others 1997), datura (Sorial and Gendy 2002), pomegranate (Saeed 2005), and oleander (Banon and others 2005).

Despite the well-defined roles of PBZ and Put in upregulating the activity of antioxidant enzymes and regulating nutrient acquisition under saline conditions in many crops, very little is known about their interactive roles in improving salt tolerance in citrus plants. Therefore, the present investigation was carried out on *Karna khatta* citrus rootstock to test the hypothesis that PBZ and Put could improve plant antioxidant metabolism and regulate accumulation of proline, sodium, chloride, potassium, and calcium in the plant.

Materials and Methods

Plant Material

The pot experiment was conducted during 2007-2008 on *Karna khatta* (*Citrus karna* Raf.) seedlings grown in a polyhouse under the following conditions: day temperature, 26-32°C; night temperature, 16-19°C; relative humidity (RH), 60-95%; and a photoperiod of 5-9 h. Seeds were collected from the citrus germplasm block of the Division of Fruits and Horticultural Technology, IARI, New Delhi. Seeds were thoroughly washed with running tap water immediately after extraction from the fruits and sown in the nursery beds. Six-month-old nucellar seedlings were selected on the basis of uniformity, vigor, leaf size, and shape. These seedlings were then transplanted in plastic pots (12 in. size) containing 5 kg of a mixture of garden soil and well-rotted farmyard manure at a ratio of 4:1. Each plant was given 15 g urea, 20 g single superphosphate, and 10 g potassium sulfate 15 days after transplanting. Garden soil had electrical conductivity $(EC)_{(1:2)} = 0.35$ dS m^{-1}, $pH_{(1:2)}$ 7.10, cation exchange capacity (CEC) = 10.65 Cmol kg^{-1}, and 0.43% organic carbon.

Treatment

The seedlings grown in pots were treated with three levels of PBZ (commercial product 'Cultar' a.i. PBZ 25% w/v), that is, 0, 250, and 500 mg L^{-1}, 30 days after transplanting. After 1 week, the required quantity of NaCl salt for 5 kg of pot soil to develop a soil EC of 3.0 dS m^{-1} was calculated as per the method suggested by Dubey and others (2006). The computed amount of NaCl salt (3.0 g kg^{-1} soil) was dissolved in 1.5 L of distilled water and poured into the pots at 2-day intervals. The EC of the soil (0.35 dS m^{-1}) was used as control. The plants were also treated with two levels of Put, that is, 0 or 50 mg L^{-1} on the same day as the NaCl treatment. The PBZ and NaCl treatments were given as a soil drench whereas Put was applied as a foliar spray. Doses of PBZ and Put were selected based on earlier works and observations of other plants (Abou El-Khashab and others 1997; Hussein and others 2006). During the experiments, control plants were irrigated with tap water (EC = 0.22 dS m^{-1}) at 2-day intervals considering the loss of moisture measured by direct weighing of the pots. The pH and EC of soil (soil: water 1:2) were observed at different intervals and the final mean pH (7.2 in nonsaline and 7.36 in saline) and the final mean salinity level (0.58 dS m^{-1} in nonsaline and 2.78 dS m^{-1} in saline) were recorded at the time of termination of the experiment, that is, 90 days after NaCl treatment.

Physiochemical Parameters

Membrane Injury Index (MII)

The method suggested by Blum and Ebercon (1981) was employed for estimation of the membrane injury index (MII) of leaves. Accurately weighed 0.1 g of freshly sampled leaf material was immersed in test tubes containing 10 ml of double-distilled water. The tubes were incubated at 45°C for 30 min in a hot water bath. Thereafter, the EC of the incubated solution (C_1) was measured with the help of a conductivity meter (Systronics India Ltd., Mumbai, India). These tubes were then incubated in a hot water bath (100°C) for 10 min. The incubated solution was cooled down to room temperature and the EC (C_2) was measured. The membrane injury index of the leaf was calculated according to the following formula: $\text{MII} = C_1/C_2$.

Relative Water Content (RWC)

The relative water content in recently matured leaves was determined following the method suggested by Brass and Wheatherly (1962). The saturated (turgid) weight of leaf discs was recorded. These leaf discs were then dried in a hot air oven at 70°C for 2-3 days until constant weight was achieved. Finally, the dry weight of the sample was recorded. The relative water content was estimated using the following formula:

$$RWC(\%) = \frac{Fresh\ weight - Oven\ dry\ weight}{Turgid\ weight - Oven\ dry\ weight} \times 100$$

Photosynthetic Rate and Stomatal Conductance

Five matured leaves from three plants in each treatment were selected and their rate of photosynthesis (μmol m^{-2} s^{-1}) and stomatal conductance (S cm^{-1}) were measured by using an infrared gas analyzer (IRGA) (LI-6200, LI-COR Biosciences, Lincoln, NE, USA).

Chlorophyll Content

The leaf chlorophyll contents (chlorophyll *a*, *b*, and total chlorophyll) were estimated using the method suggested by Hiscox and Israelstam (1979). Accurately weighed 100 mg of clean, fully matured leaves were immersed in 10 ml of dimethylsulfoxide (DMSO) (AR grade, SRL Chem. Co., Mumbai, India). The sample was incubated at 70°C for 4 h in an incubator (TH 7004, Sanco Co., New Delhi). After incubation the sample was removed and 1 ml of the solution was diluted to 5 ml with pure DMSO and the sample was read on a UV–VIS spectrophotometer (UV–VIS 5704SS, E. C. India Limited, Hyderabad, India) at 645 and 663 nm using pure DMSO as a blank. Using a standard formula, chlorophyll *a*, *b*, *a*/*b* ratio, and total chlorophyll were estimated.

Proline Analysis

The proline content in the matured leaves of each treatment was estimated using a rapid colorimetric method as suggested by Bates and others (1973). A fresh leaf (0.5 g) was homogenized in a prechilled mortar and pestle with 5 ml of 3% sulfosalicylic acid. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was diluted to 10 ml with double-distilled water. Then 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 tube was heated for 1 h at 100°C in a hot water bath. The reaction was terminated by keeping the solution in an ice bath followed by addition of 4 ml of toluene and stirred vigorously for 20-30 s. The chromophore-containing 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 Chem Co., Mumbai, India).

Antioxidant Enzyme Activity

The activity of SOD in a leaf sample was determined by the method proposed by Fridovich (1975). The assay is based on the ability of SOD to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture was prepared in tubes containing 0.2 ml of 200 mM methionine, 0.2 ml of 1.5 mM EDTA, 0.2 ml of 1.125 mM NBT, 0.2 ml of 75 μM riboflavin, 0-10 μl of enzyme extract and phosphate buffer (50 mM; pH 7.8) to make the final volume of the reaction mixture to 3 ml. Riboflavin was added as the last component and the tubes were shaken well. The reaction was started for a specified time of 15 min by keeping the tubes 30 cm below a light bank consisting of two 15-W fluorescent lamps. After 15 min, the light was switched off and the tubes were immediately covered with a black cloth to stop the reaction. The absorbance of the mixture was read at 560 nm on a UV–VIS spectrophotometer. A complete reaction mixture, lacking enzyme, developing maximum color served as a control. A unirradiated complete reaction mixture with no color development served as a blank. The absorbance (log A_{560}) was plotted as a function of the volume of enzyme extract in the reaction mixture. The volume of enzyme extract resulting in 50% reduction in absorbance in comparison to that of the control was read from the resultant

graph. One unit of SOD activity in the sample was taken as the amount of enzyme that caused a 50% reduction in the absorbance compared with the control tube lacking enzyme extract. Finally, the SOD was quantified on the basis of soluble protein content of the sample and expressed as mg^{-1} protein min^{-1}.

The method suggested by Luck (1965) was followed to estimate the catalase activity in the plant sample. The assay is based on the estimation of residual hydrogen peroxide (H_2O_2) by oxidation with potassium permanganate ($KMnO_4$) by titration. The reaction mixture was prepared in tubes by adding 3 ml of phosphate buffer (0.1 M; pH 7.0), 2 ml of H_2O_2 (5 mM), and 1 ml of enzyme extract. The reaction mixture was incubated for 1 min at 20°C followed by the addition of 10 ml of H_2SO_4 (0.35 M) to stop the reaction. A blank was prepared by adding enzyme extract to an acidified solution of reaction mixture at zero time. The residual H_2O_2 was estimated by titrating the reaction mixture against $KMnO_4$ (0.01 M) until the appearance of a faint pink color persisted for at least 15 s. Catalase activity was expressed as µmol H_2O_2 hydrolyzed mg^{-1} protein min^{-1}.

The activity of peroxidase in leaf samples was determined by the method proposed by Thomas and others (1981). The assay utilizes guiacol as the substrate. The reaction mixture was prepared in tubes by adding 3 ml of phosphate buffer (0.1 M; pH 7.0), 30 µl of H_2O_2 (20 mM), 50 µl of guiacol (20 mM) as enzyme substrate, and 50 µl of enzyme extract. The reaction mixture was incubated in cuvettes for exactly 10 min at room temperature. The absorbance was read at 436 nm on a UV–VIS spectrophotometer. A increase in absorbance was recorded at 30-s intervals until a constant reading was obtained. Peroxidase activity was expressed as number of absorbance units g^{-1} fresh weight (FW) of leaf.

Tissue Nutrients

Total sodium and potassium contents in plant leaves were estimated from the diacid-digested plant sample using the method proposed by Jackson (1980) that uses a microprocessor-based flame photometer (Flame Photometer-128, Systronics India, Ahmedabad, Gujarat, India). Calcium content in leaves was determined by an AVANTA atomic absorption spectrophotometer (GBC Scientific Equipment Pty Ltd., Dandenong, VIC, Australia) as per the method outlined by Jackson (1980).

Chloride content in the leaves was quantified by the mercury (II) thiocyanate method as suggested by Adriano and Doner (1982). However, chloride extraction from the plant samples was done with 0.1 M sodium nitrate in 1:100 ratio (Gaines and others 1984). The solutions were shaken in a horizontal mechanical shaker for 5 min. The solution was filtered through Whatman No. 1 filter paper and used for colorimetric determination of chloride. The reaction mixture consisted of 10 ml of sample extract and 2 ml of ferric (III) nitrate nanohydrate solution and saturated mercury (II) thiocyanate solution. The mixture was kept for 10 min and the volume made to 25 ml. It was mixed properly and the bright brick color that developed was read at 460 nm wavelength on a UV–VIS spectrophotometer.

Statistical Analysis

The experiment was conducted in a completely randomized block factorial design with five replications, two salinity levels, three PBZ concentrations, and two levels of putrescine. The data were analyzed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA) for the calculation of F values. Significance of variance was estimated by applying the F test at the 5% level of significance.

Results

The MII increased 43.75% in salinized plants over nonsalinized plants (Table 1). In the presence of NaCl, the maximum MII was recorded in salinized seedlings without PBZ or Put, whereas the minimum (45.27% lower) as compared to control was measured with the application of 250 mg L^{-1} PBZ (Fig. 1a).

The maximum reduction in RWC (4.15%) occurred in salinized seedlings compared to nonsalinized controls (Table 1). Interaction among salinity, PBZ, and putrescine showed that RWC was enhanced by 1.75% in the nonsaline condition and 17.33% in saline conditions with the application of 250 mg L^{-1} PBZ alone (Fig. 1b). Furthermore, all the treatments improved RWC in both nonsaline and saline conditions compared to the RWC in their respective controls.

The photosynthetic rate was reduced under salinized conditions. PBZ and Put had significant effects on the photosynthetic rate (Table 1). Seedlings treated with 50 mg L^{-1} Put alone yielded a 67.91% higher photosynthetic rate in nonsaline conditions. However, in saline conditions, application of 250 mg L^{-1} PBZ yielded an 80% higher photosynthetic rate, which was statistically at par with the application of 50 mg L^{-1} Put alone or with 500 mg L^{-1} PBZ (Fig. 1c).

Stomatal conductance was at a minimum in salinized seedlings. Furthermore, the mean effect of PBZ and Put was improvement in stomatal conductance (Table 1). The maximum stomatal conductance (104.34% higher) compared to that of controls occurred with the application of 500 mg L^{-1} PBZ alone under saline conditions.

Table 1 Effect of salinity, paclobutrazol (PBZ), and putrescine (Put) on membrane injury index (MII), relative water content (RWC), photosynthetic rate, chlorophyll fractions, antioxidant enzymes, and nutrient acquisition in salt-susceptible citrus rootstocks *Karna khatta*

Parameters	Saline	Nonsaline	P_0	P_1	P_2	T_0	T_1	$p \leq 0.05$		
								Salinity	PBZ	Put
MII	0.207	0.144	0.177	0.153	0.198	0.173	0.178	*	*	ns
RWC	91.09	95.03	91.84	93.70	93.63	92.38	93.74	*	ns	ns
Photosynthetic rate	2.74	2.88	2.70	2.73	3.01	2.91	2.72	*	*	ns
Stomatal conductance	0.28	0.37	0.33	0.35	0.29	0.35	0.30	*	*	*
Chlorophyll *a*	0.918	1.602	1.211	1.348	1.221	1.305	1.214	*	*	*
Chlorophyll *b*	0.195	0.261	0.223	0.191	0.270	0.183	0.272	*	*	*
Total chlorophyll	1.112	1.862	1.432	1.538	1.492	1.488	1.486	*	*	ns
Chlorophyll *a*/*b* ratio	6.37	6.66	7.32	7.56	4.68	8.32	4.69	*	*	*
SOD in leaf	25.00	21.91	21.33	24.00	25.04	22.96	23.95	*	*	*
Catalase in leaf	12.21	7.93	10.20	10.42	9.59	10.18	9.96	*	*	*
Peroxidase in leaf	3.58	2.37	2.67	3.25	3.00	2.88	3.07	*	*	*
Proline in leaf	150.96	111.04	125.00	137.22	130.78	122.30	139.70	*	*	*
Na (%) in leaf	0.807	0.488	0.666	0.617	0.660	0.661	0.634	*	*	*
Cl (%) in leaf	1.307	0.998	1.238	1.094	1.126	1.172	1.134	*	*	ns
K (%) in leaf	1.79	1.87	1.81	1.81	1.88	1.78	1.89	*	*	*
Ca (%) in leaf	1.24	2.60	1.74	1.99	2.03	1.87	1.97	*	*	*

$P_0 = 0.0$ mg L^{-1} PBZ, $P_1 = 250$ mg L^{-1} PBZ, $P_2 = 500$ mg L^{-1} PBZ, $T_0 = 0.0$ mg L^{-1} Put, $T_1 = 50$ mg L^{-1} Put

Interestingly, the combined application of PBZ and Put reduced stomatal conductance under saline conditions (Fig. 1d).

Chlorophyll *a* and *b* were reduced significantly by salinity. Application of PBZ and Put had a significant effect on chlorophyll *a* (Table 1). In the absence of NaCl, the highest chlorophyll *a* content (31.17% more) was observed with the combined application of 250 mg L^{-1} PBZ and 50 mg L^{-1} Put (Fig. 2a). However, in salinized seedlings, the maximum chlorophyll *a* content was found when plants were treated with 500 mg L^{-1} of PBZ alone (2.03% higher) compared to salinized controls. The maximum chlorophyll *b* content was recorded when salinized plants were treated with 50 mg L^{-1} Put alone followed by the combined treatment of 500 mg L^{-1} PBZ and 50 mg L^{-1} Put (Fig. 2b).

Salt stress decreased total chlorophyll content. However, application of PBZ improved total chlorophyll content (Table 1). Total chlorophyll content increased in both nonsalinized and salinized conditions with the application of PBZ or Put. In the absence of salt stress, total chlorophyll was 23.12% higher in seedlings treated with the combined application of 250 mg L^{-1} PBZ and 50 mg L^{-1} Put (Fig. 2c). However, under salt stress, the maximum total chlorophyll content was recorded with the application of 500 mg L^{-1} PBZ. The chlorophyll *a*/*b* ratio was reduced under salt stress (Table 1). The application of PBZ or Put or the application of 250 mg L^{-1} PBZ and 50 mg L^{-1} Put combined enhanced the chlorophyll *a*/*b* ratio in the absence of NaCl stress. Compared with salinized controls, the application of either of the treatments reduced the chlorophyll *a*/*b* ratio (Fig. 2d).

Superoxide dismutase activity (SOD) was significantly enhanced under salinity (Table 1). Moreover, the application of PBZ and Put further improved SOD activity under both salinized and nonsalinized conditions. Under nonsalinized conditions, the SOD level increased by 39.74% with the combined treatment of 500 mg L^{-1} PBZ and 50 mg L^{-1} Put compared to controls. Application of the same combination treatment increased SOD activity by 10.5% in salinized seedlings (Fig. 3a).

Catalase activity of seedlings increased significantly (53.97%) under salt stress compared to that of nonsalinized seedlings. In the absence of NaCl, the application of 250 mg L^{-1} PBZ or 50 mg L^{-1} Put or both improved catalase activity. Furthermore, it was noted that the application of a higher dose of PBZ alone or in combination with Put reduced catalase activity (Fig. 3b). Under salt stress, PBZ or Put alone or in combination further improved catalase activity. The maximum increase (30.33%) was observed with the application of 250 mg L^{-1} PBZ alone (Fig. 3b). The combined application of PBZ and Put lowered catalase activity compared to PBZ or Put alone in both saline and nonsaline conditions.

Salinity increased peroxidase activity by 51.11% over nonsaline conditions (Table 1). Furthermore, the application of either PBZ or Put or both increased peroxidase activity under both saline and nonsaline conditions. In the

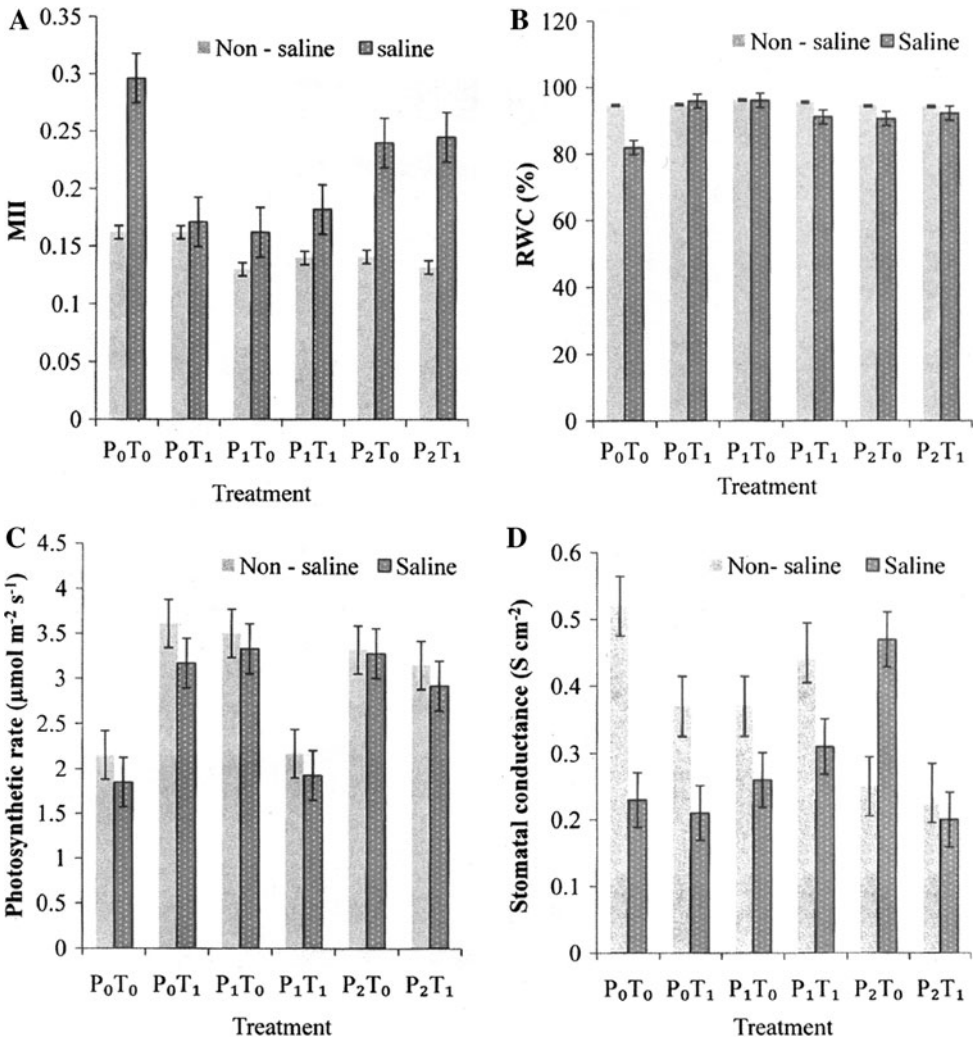

Fig. 1 Effect of salinity (NaCl), paclobutrazol (PBZ), and putrescine (Put) on membrane injury index (MII) (**a**), relative water content (RWC) (**b**), photosynthetic rate (**c**), and stomatal conductance (**d**) in *Karna khatta* citrus rootstock. There was a significant interaction among salinity, PBZ, and Put ($p \leq 0.05$). The LSDs ($p \leq 0.05$) for interaction among salinity, PBZ, and Put were MII = 0.048, RWC = 3.42, photosynthetic rate = 0.95, stomatal conductance = 0.02. $n = 5$ replicates. $P_0 = 0.0$ mg L^{-1} PBZ, $P_1 = 250$ mg L^{-1} PBZ, $P_2 = 500$ mg L^{-1} PBZ, $T_0 = 0.0$ mg L^{-1} Put, $T_1 = 50$ mg L^{-1} Put

absence of salt stress, the application of 250 mg L^{-1} PBZ resulted in a higher peroxidase activity (50.77%) compared to that of controls. However, under salt stress, the combined application of 250 mg L^{-1} PBZ and 50 mg L^{-1} Put increased peroxidase activity by 21.42% compared to that of salinized controls (Fig. 3c).

Proline content increased under salt stress compared to that of controls. Interaction among salinity, PBZ, and Put showed that in nonsalinized seedlings the maximum proline content (28.82% more) was recorded with a combined treatment of 500 mg L^{-1} PBZ and 50 mg L^{-1} Put. Under saline conditions, the highest proline content (28.67% higher) was observed with the combined treatment of 250 mg L^{-1} PBZ and 50 mg L^{-1} Put (Fig. 3d).

Sodium content in leaves was affected significantly by salinity, PBZ, and Put (Table 1). In the absence of NaCl, the minimum Na^+ accumulation was recorded with the treatment of 250 mg L^{-1} PBZ and 50 mg L^{-1} Put. However, in salinized seedlings, the application of 250 mg L^{-1} PBZ was more effective in reducing Na^+ accumulation in leaf tissues (Fig. 4a).

Cl^- content in the leaves of salinized seedlings was increased by 30.96% compared to that of controls (Table 1). Application of PBZ or Put alone or in combination had a significant effect on Cl^- accumulation (Table 1). Under nonsalinized treatment, the application of either 50 mg L^{-1} Put or 250 mg L^{-1} PBZ reduced the leaf Cl^- concentration by 6.75% compared to that of controls, whereas in salinized seedlings the lowest leaf Cl^- concentration was found with the application of 250 mg L^{-1} PBZ alone. Furthermore, it was noted that all the treatments reduced Cl^- accumulation in leaf tissues compared to the salinized control (Fig. 4b).

Salinization decreased the accumulation of K^+ in leaf tissues. Leaf K^+ content was higher in PBZ or Put-treated seedlings (Table 1). Interaction among salinity, PBZ, and Put showed that the leaf K^+ content was maximum when the plants were sprayed with 50 mg L^{-1} Put alone. However, in salinized seedlings the leaf K^+ content increased

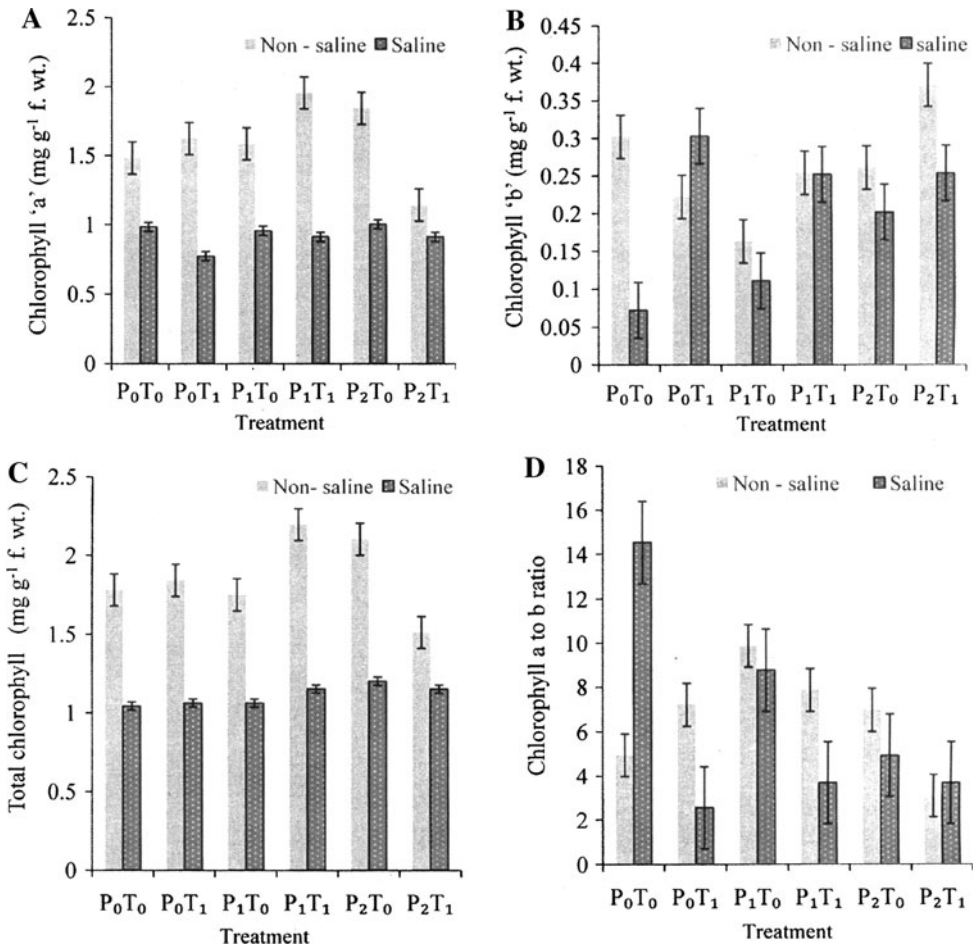

Fig. 2 Effect of salinity (NaCl), paclobutrazol (PBZ), and putrescine (Put) on chlorophyll *a* (**a**), chlorophyll *b* (**b**), total chlorophyll (**c**), and chlorophyll *a*/*b* ratio (**d**) in *Karna khatta* citrus rootstock. There was a significant interaction among salinity, PBZ, and Put ($p \leq 0.05$). The LSDs ($p \leq 0.05$) for interaction among salinity, PBZ, and Put were chlorophyll $a = 0.05$; chlorophyll $b = 0.021$; total chlorophyll $= 0.03$; chlorophyll *a*/*b* ratio $= 0.68$. $n = 5$ replicates. $P_0 = 0.0$ mg L^{-1} PBZ, $P_1 = 250$ mg L^{-1} PBZ, $P_2 = 500$ mg L^{-1} PBZ, $T_0 = 0.0$ mg L^{-1} Put, $T_1 = 50$ mg L^{-1} Put

by 71.77% when plants were treated with 500 mg L^{-1} PBZ alone (Fig. 4c).

Salinity reduced Ca^{2+} by 52.31% compared to that of control seedlings. Both PBZ and Put also had a significant effect on leaf Ca^{2+} content (Table 1). Interaction among salinity, PBZ, and Put revealed that the maximum increase (19.57%) was found when seedlings were treated with 500 mg L^{-1} PBZ under nonsalinized conditions, whereas in the presence of NaCl, the combined application of 500 mg L^{-1} PBZ and 50 mg L^{-1} Put resulted in the highest concentration of Ca^{2+} in leaf tissues (Fig. 4d).

Discussion

The results of this study demonstrate a marked reduction in relative water content and higher membrane injury index under saline conditions. Furthermore, the application of PBZ or Put alone or in combination improved RWC and reduced MII in salt-sensitive *Karna khatta* under salt stress. PBZ is reported to decrease water loss and increase tolerance of plants to water stress (Fletcher and Nath 1984). This is probably due to the fact that triazole compounds like PBZ reduce GA biosynthesis and increase ABA and cytokinin contents, which ultimately help in maintaining better water balance in the plants. The findings of the present study agree with the results on PBZ-mediated stress tolerance observed by several authors (Upadhyaya and others 1989; Jinelle and Fletcher 1996). According to Tang and Newton (2005) also, polyamines (PAs) (mainly Put) reduce salt-induced oxidative damage.

Zekri (1991) reported a reduction in stomatal conductance under saline conditions in sour orange and Cleopatra mandarin. It is also possible that high amounts of Na^+ decrease the osmotic potential of guard cells (Sairam and others 1995), causing stomatal closure (Behboudian and others 1986). Salinity causes a reduction in photosynthesis through its adverse impact on gas exchange parameters such as stomatal conductance and stomatal resistance. Our results suggest that the application of PBZ or Put alone or in combination improved the photosynthetic rate and stomatal conductance in salt-susceptible *Karna khatta* under saline conditions. The ability of PBZ and Put to ameliorate the negative effects of salt stress on photosynthesis may be due to the reduced uptake of Na^+ and Cl^- and improvement in the concentrations of K^+ and Ca^{2+} in leaf tissues.

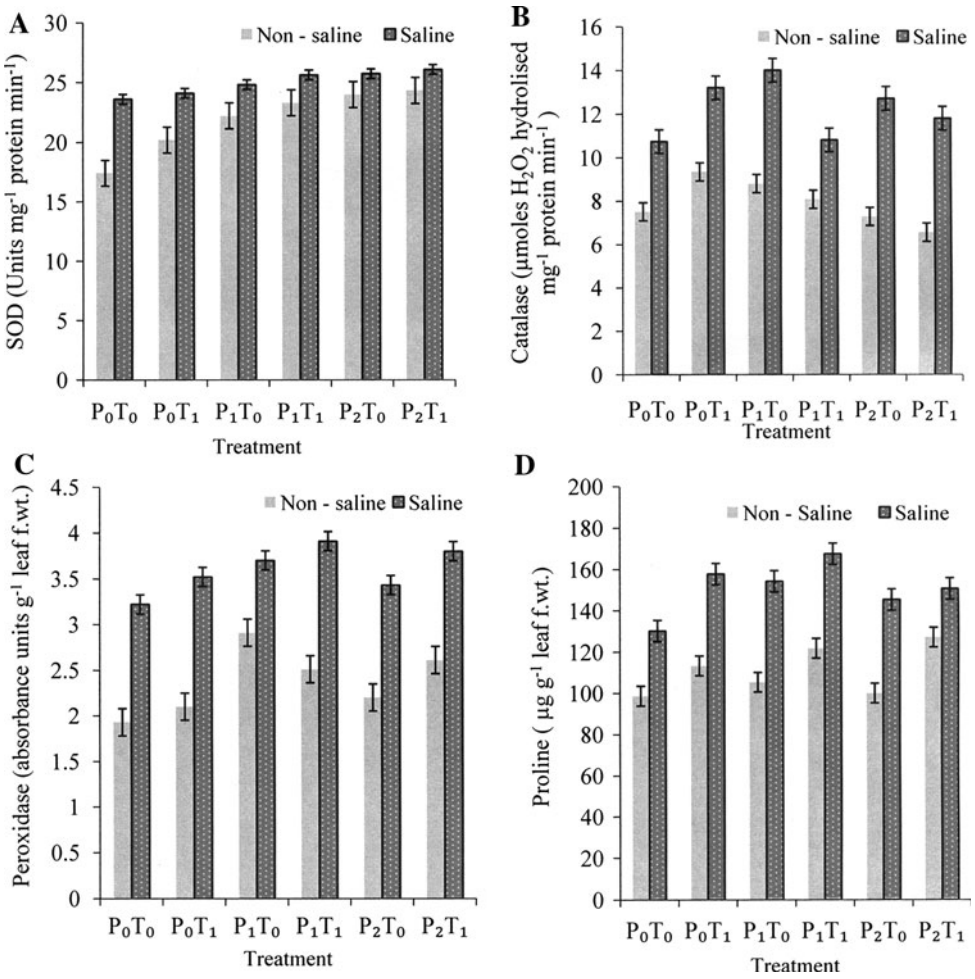

Fig. 3 Effect of salinity (NaCl), paclobutrazol (PBZ), and putrescine (Put) on superoxide dismutase (SOD) (**a**), catalase (**b**), peroxidase (POD) (**c**), and proline (**d**) in *Karna khatta* citrus rootstock. There was a significant interaction among salinity, PBZ, and Put ($p \leq 0.05$). The LSDs ($p \leq 0.05$) for interaction among salinity, PBZ, and Put were SOD = 0.05; catalase = 0.47; POD = 0.23; proline = 1.48. $n = 5$ replicates. $P_0 = 0.0$ mg L^{-1} PBZ, $P_1 = 250$ mg L^{-1} PBZ, $P_2 = 500$ mg L^{-1} PBZ, $T_0 = 0.0$ mg L^{-1} Put, $T_1 = 50$ mg L^{-1} Put

In the present study, it was noticed that Put improved the photosynthetic rate, even in the absence of NaCl stress. However, exogenous application of Put reduced the stomatal conductance in saline and nonsaline conditions. Although, Ndayiragije and Lutts (2007) reported improvement in net photosynthesis in response to Put treatment in rice, the situation appeared to be different for citrus, and our data corroborate previous findings demonstrating that Put induced stomatal closure in wheat which exhibited high water content (Liu and others 2000).

A difference in reduction of chlorophyll under saline conditions in different citrus species was also reported by Nieves and others (1991). Application of PBZ alone or in combination with Put resulted in total chlorophyll content higher than their respective controls. PBZ increased chlorophyll content may be partly due to the increase in the mass of the root system (data not shown), which is the major site of cytokinin biosynthesis (Sopher and others 1999). The increase in cytokinin levels was associated with stimulated chlorophyll biosynthesis (Fletcher and Arnold 1986). Zeid (2004) had also reported improvement in photosynthetic pigment by the application of Put.

Salinity has been reported to increase SOD activity in pea plants (Hernandez and others 2000) and in citrus (Almansa and others 2002). Catalase is an important antioxidant enzyme that catabolizes hydrogen peroxide (Larsen and others 1988). Application of PBZ alone or in combination with Put enhanced the activities of SOD, catalase, and peroxidase under saline and nonsaline conditions. Excess ROS may initiate membrane lipid peroxidation, weaken membrane lipid unsaturation, trigger membrane protein polymerization, and result in an increase in membrane permeability (Chen 1991). The results of the present work suggest that the application of PBZ or Put alone or in combination improved antioxidant systems like SOD, catalase, and peroxidase to repair the damage caused by ROS. Increasing activities of SOD, catalase, and peroxidase with the application of PBZ and Put proved the role of these plant growth regulators in mitigating the adverse effects of salt stress in citrus. The data of the present investigation are consistent with the hypotheses of Fletcher and Hofstra (1990), which suggested that triazoles-induced stress tolerance in plants may be due, at least in part, to increased antioxidant activity.

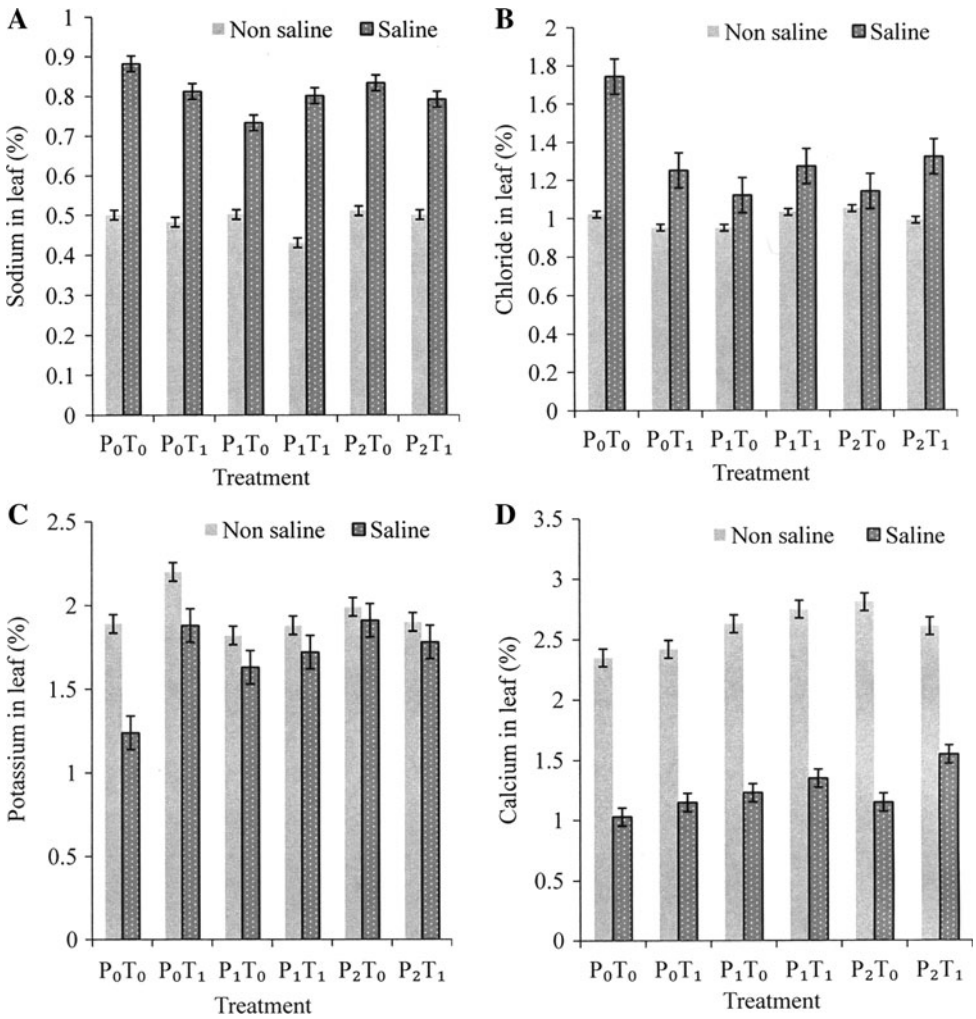

Fig. 4 Effect of salinity (NaCl), paclobutrazol (PBZ), and putrescine (Put) on sodium content in leaf (**a**), chloride content in leaf (**b**), potassium content in leaf (**c**), and calcium content in leaf (**d**) of *Karna khatta* citrus rootstock. There was a significant interaction among salinity, PBZ, and Put ($p \leq 0.05$). The LSDs ($p \leq 0.05$) for interaction among salinity, PBZ, and Put were sodium in leaf = 0.05; chloride in leaf = 0.13; potassium in leaf = 0.05; calcium in leaf = 0.52. $n = 5$ replicates. $P_0 = 0.0$ mg L^{-1} PBZ, $P_1 = 250$ mg L^{-1} PBZ, $P_2 = 500$ mg L^{-1} PBZ, $T_0 = 0.0$ mg L^{-1} Put, $T_1 = 50$ mg L^{-1} Put

Application of PBZ or Put alone or in combination elevated the level of proline accumulation under both nonsaline and saline conditions. Proline is the key osmolyte contributing to osmotic adjustment (Yoshiba and others 1997). It can also improve stress tolerance by protecting and stabilizing membranes and enzymes during stress conditions (Rudoplh and others 1986). Increasing levels of proline by the application of PBZ and/or Put provided better osmotic adjustment and also helped stabilize membrane and enzymes during stress conditions. Bagga and others (1997) suggested that under salt stress Put catabolism (via diamine oxidase) could contribute to proline accumulation. Similar results were also obtained by Verma and Mishra (2005), Zhao (2004), and Tang and Newton (2005), who had also reported that the application of Put increased the activities of antioxidant enzymes, thereby decreasing lipid peroxidation under salt stress.

One of the primary plant responses to salinity is an influx of Na^+ and Cl^- and a decrease of K^+ concentration in plant tissues. It is evident that one of the mechanisms by which plants tolerate salt stress depends upon their capacity to maintain a lower Na^+ and Cl^- influx and higher K^+ concentration (Shannon and Grieve 1999). In the present experiment, the results showed that NaCl treatment resulted in an increase in Na^+ and Cl^- concentrations and a reduction in K^+ concentration in leaf tissues. Recently, evidence indicated that Put and/or PBZ played an important role in plant responses to environmental stresses. In our experiment, PBZ and/or Put inhibited reduction of K^+ and Ca^{2+} concentrations and increases of Na^+ and Cl^- concentrations resulting in a higher K^+/Na^+ ratio in leaf tissues under NaCl stress. This is consistent with other reports that suggested that exogenously applied PAs could regulate ion homeostasis and absorption and translocation of toxic ions in crops such as Put in rice (Krishnamurthy 1991), cucumber (Suping and others 2007), and bean (Zeid 2004), Put and spermidine (Spd) in wheat (Iqbal and Asraf 2005), and PBZ in wheat (Hajihashemi and others 2007) and strawberry (Jamalian and others 2008).

In conclusion, salt stress can result in an increase in Na^+ and Cl^- concentrations and in Na^+/K^+ and Na^+/Ca^{2+} ratios concomitant with a decrease in K^+ and Ca^{2+}

accumulations. Application of PBZ and/or Put may have a positive impact on cation discrimination in the process of absorption and transport; regulation of the absorption and translocation of Na^+, Cl^-, K^+, Ca^{2+}; and alleviation of the deleterious effects of salt stress by improving the activities of antioxidant enzymes like SOD, catalase, and peroxidase in citrus rootstock *Karna khatta*. Furthermore, the application of PBZ (250 mg L^{-1}) was found more effective in mitigating salt-induced negative effects.

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