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Physiological and biochemical responses of citrus rootstocks under salinity stress

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ABSTRACT

Physio-biochemical response of indigenous citrus rootstocks namely Attanni-1 (*Citrus rugulosa*), Attanni-2 (*C. rugulosa*) and *Jatti khatti* (*C. jambhiri*) under NaCl stress was studied. The experiment was performed on 1-year-old potted plants which were irrigated with non-saline and saline (25 and 50 mM NaCl) water. Results indicated that salt stress had a pronounced effect on different physiological and biochemical parameters of these rootstocks. The highest MII (0.159) was recorded in *Jatti khatti* under 50 mM salinity followed by Attani-2 and Attani-1. There was almost two-fold reduction in RWC in the *Jatti khatti* rootstock under 50 mM salinity as compared to Attani-1 and Attani-2 rootstocks. The maximum (2.22 mg g^{-1} FW) total chlorophyll was recorded in *Jatti khatti* (control) followed by non-salinised Attani-1 and Attani-2. Salt stress induced a sharp reduction in total chlorophyll content in *Jatti khatti* as compared to other two rootstocks. The highest SOD (45.67 units mg^{-1} protein min^{-1}) and CAT (5.34 μ moles H_2O_2 hydrolized mg^{-1} protein min^{-1}) activities were recorded in the *Jatti khatti* under 50 mM NaCl salinity followed by Attani-2 and Attani-1. CAT activity declined in different rootstocks at 50 mM salinity. There was a sharp increase in salt induced POD levels in different rootstcoks upto 25 mM salinity with the highest activity (0.75 Absorbance units g^{-1} FW) in *Jatti khatti* followed by Attani-2 rootstocks. Based on observations on membrane stability, relative water content, photosynthetic pigments, proline content and activity of antioxidant enzymes, the relative salt tolerance of citrus rootstocks was adjudged to decrease in the following order: Attanni-1>Attanni-2> *Jatti khatti*.

Key words: Antioxidant enzymes, chlorophyll, citrus, proline, rootstocks, salinity.

INTRODUCTION

Among different biotic and abiotic stresses affecting citrus cultivation, soil and water salinity are the major impediment as majority of the citrus species and cultivars are characterized as sensitive to salt stress. The high salt susceptibility of citrus is substantiated by the fact that fruit yield decreases by about 13% for every 1 dSm^{-1} increase in salinity above 1.43 dSm^{-1}, which has been reported as threshold value of soil saturation post salinity for *Citrus* spp. Notwithstanding this observation, substantial differences in salt tolerance of different scion and rootstock genotypes have also been reported. The salinity induced changes in growth, physiological and biochemical parameters manifested as toxic accumulations of Na^+ and Cl^- ions, impaired water relations, osmotic stress and poor photosynthetic efficiency account for poor plant performance in salt affected soils (Murkute *et al.*, 14). High salt concentration in the root zone reduces soil water potential and availability of water. Besides these deleterious effects, salinity triggers the generation of reactive oxygen species such as hydroxyl radicals (OH) and superoxide anions (O_2^-) which impair plant metabolism by oxidative damage of lipids, proteins and nucleic acids. To mitigate these adverse effects, plants activate different enzymatic antioxidant defense systems like superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Wu *et al.*, 18). Since majority of the commercial citrus fruits are budded onto rootstocks, their salt tolerance seems to be associated with the ability of the root system to restrict the uptake and/or transport to Na^+ and Cl^- ions to shoots (Levy and Syverstsen, 12). The selection of salt tolerant rootstocks is, thus, a promising strategy for mitigating the salinity stress in commercial scion cultivars. The identification of salt tolerant genotypes can also facilitate their inclusion in future citrus improvement programmes for developing salt tolerant scion varieties and rootstocks. Although the effects of salt stress in different citrus varieties and rootstocks are well documented, the work on indigenous rootstocks is by and large scanty. In this backdrop, we investigated the physio-biochemical relations of three indigenous citrus rootstocks to determine the differences in and the physiological bases of salt tolerance for appraising their suitability for use as rootstocks in moderately

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salt-affected soils as well as parents in future breeding programmes.

MATERIALS AND METHODS

The experiment was performed with 1-year-old potted rootstocks of Attanni-1 (*Citrus rugulosa*), Attanni-2 (*C. rugulosa*) and *Jatti khatti* (*C. jambhiri*). The uniform sized one-year-old seedling rootstocks were selected and transplanted from nursery to plastic pots (10 inches size) containing 5 kg mixture of garden soil and well-rotted farmyard manure (4:1). Each plant was given 15 g urea, 20 g single superphosphate, and 10 g potassium sulfate after transplanting. The electrical conductivity ($EC_{1:2}$) and pH values of the experimental soil (0.39 dS m^{-1} and 7.2, respectively) and control irrigation water (0.22 dS m^{-1} and 7.34, respectively) were determined before start of the experiment. The experiment was laid out in factorial randomized block design (RBD) with three replications. The plants were irrigated twice a week for 30 days (8 applications) with normal (0.22 dS m^{-1}) and saline (25 and 50 mM) water. After 30 days of treatment, the leaves were analysed for estimating salinity induced physio-biochemical changes. The membrane injury index (MII) was estimated by the method of Blum and Ebercon (4), whereas, the method suggested by Barrs and Wheatherly (2) was employed to estimate the relative water content (RWC). The leaf chlorophyll contents (chlorophyll 'a', 'b', and total chlorophyll) were estimated using the method of Hiscox and Israelstam (9). The proline content was determined by rapid colorimetric method (Bates *et al.*, 3). The leaf samples from each treatment were collected freshly in ice box to prevent the proteolytic activity. One g of clean leaf sample was taken and homogenized in a pre-chilled mortar and pestle with 5 ml of chilled phosphate buffer (50 mM; pH 7.0). The homogenate was collected in oak-ridge centrifuge tubes and centrifuged at 15,000 rpm for 20 min. at 4°C. The supernatant so obtained was sieved through two layers of muslin cloth and stored in refrigerator for use in the estimation of anti-oxidant enzymes activity. Superoxide dismutase (SOD) in leaf samples was determined by the method proposed by Fridovich (6). The method suggested by Luck (13) was followed to estimate the catalase (CAT) activity. The activity of peroxidase (POD) in leaf samples was determined by the method proposed by Thomas *et al.* (17). The data were analyzed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA) for the calculation of F values.

RESULTS AND DISCUSSION

Indigenous citrus rootstocks showed significant differences for membrane injury index and relative water content (Table 1). The lowest (0.159) MII was recorded in Attani-1, whereas, the highest (0.242) membrane injury occurred in *Jatti khatti*. The mean effect of salinity was also found to be significant with the lowest MII recorded in control plants, while the maximum in plants irrigated with 50 mM water. Regarding the interaction effect of rootstock and salinity level, the lowest MII was estimated in Attani-1 plants under control, which exhibited non-significant differences with the non-salinized Attani-2 and *Jatti khatti* plants. The highest MII was recorded in *Jatti khatti* under 50 mM salinity followed by Attani-2 and Attani-1 under the same treatment. Our observation that salt stress caused differential membrane injuries in citrus rootstocks, with more pronounced effects in *Jatti khatti*, is in accordance with earlier findings of Dubey *et al.* (5) who also reported differences in salinity induced membrane damage in salt tolerant and susceptible citrus rootstocks. Under stress conditions, plasmalemma and lipid membrane are damaged and these alterations in membrane integrity can lead to increased cell permeability and electrolyte leakage (Blum and Ebercon, 4). The observed differences in membrane stability could also be due

Table 1. Effect of water salinity on membrane injury index (MII) and relative water content (RWC) in citrus rootstocks.

Rootstock	Membrane injury index				Relative water content (%)			
Salinity →	Control	25 mM	50 mM	Mean	Control	25 mM	50 mM	Mean
Attani-1	0.12	0.16	0.19	0.159	97.23	94.26	93.26	94.92
Attani-2	0.13	0.19	0.23	0.186	97.36	93.63	91.82	94.27
Jatti khatti	0.13	0.23	0.36	0.242	97.32	94.16	87.02	92.83
Mean	0.13	0.19	0.26		97.30	94.02	90.70	
$CD_{0.05}$								
Rootstock (R)		0.011				0.76		
Salinity (S)		0.011				0.76		
R × S		0.019				1.32		

to differences in plasma membrane composition of the citrus rootstocks. This signifies the importance of a particular plasma membrane lipid class in stress protection. This character needs to be investigated in detail for the better understanding of salt tolerance in citrus.

Among rootstocks, the highest (94.92%) RWC was recorded in Attani-1 which was statistically at par with Attani-2 (94.7%), but statistically significant as compared to *Jatti khatti* (92.83%). The mean effect of salinity was also found to be significant and the highest RWC was recorded in control plants followed by 25 and 50 mM salinity levels. The rootstock and salinity interactions exhibited a non-significant difference between control and 25 mM salinity levels, whereas significant differences among three rootstocks were recorded at 50 mM salinity with the highest (93.26%) and the lowest (87.02%) RWC values obtained in Attani-1 and *Jatti khatti* plants, respectively. There was almost two-fold reduction in RWC in the *Jatti khatti* rootstock under 50 mM salinity as compared to Attani-1 and Attani-2 rootstocks, which exhibited significantly less reductions. The decrease in RWC seems related to high salt concentration of the external solution, which causes osmotic stress and dehydration at the cellular level (Greenway and Munns, 8). Earlier research on citrus (Patel *et al*., 16) have also demonstrated progressive reductions in leaf RWC with increasing salinity.

Irrespective of salinity levels, the highest (1.48 mg g^{-1} FW) chlorophyll 'a' content was recorded in *Jatti khatti* followed by Attani-2 (1.34 mg g^{-1} FW) and Attani-1 (1.28 mg g^{-1} FW) plants. Among the salinity levels, the highest chlorophyll 'a' content was noted in control plants followed by 25 and 50 mM salinity levels. The interaction effects of rootstock and salinity level showed maximum chlorophyll 'a' content in *Jatti khatti* plants under control, which was significantly higher as compared to the non-salinized Attani-2 and Attani-1 plants. Notwithstanding the higher chlorophyll 'a' contents recorded in *Jatti khatti* leaves as compared to other two rootstocks, salinity caused a sharp reduction in chlorophyll 'a' in *Jatti khatti*. At 50 mM salinity, there was 35.86% reduction over control in *Jatti khatti*, whereas the corresponding values for Attani-1 and Attani-2 rootstocks were 14.28 and 13.10%, respectively (Table 2).

All three rootstocks, irrespective of salinity level, exhibited significant differences in chlorophyll 'b' values with the highest (0.31 mg g^{-1} FW) chlorophyll 'b' content recorded in *Jatti khatti* significantly followed by Attani-1 and Attani-2 plants. Significant differences were also observed among the salinity levels with the highest chlorophyll 'b' content noted in control plants followed by 25 and 50 mM salinity levels, regardless of rootstocks. The interaction effects of rootstock and salinity level on chlorophyll 'b' contents were found to be non-significant (Table 2).

Total chlorophyll content in citrus rootstocks exhibited a trend similar to chlorophyll 'a'. The mean effect of rootstock was found to be significant with the highest total chlorophyll content in *Jatti khatti* followed by Attani-1 and Attani-2. The mean effect of salinity was also found to be significant. The highest total chlorophyll was in control plants followed by 25 and 50 mM salinity levels. The interaction effects of rootstock and salinity level revealed that the maximum (2.22 mg g^{-1} FW) total chlorophyll was recorded in *Jatti khatti* (control) followed by Attani-1 (control) and Attani-2 (control) plants. Contrary to the higher values obtained in control plants, salt stress induced a sharp reduction in total chlorophyll content in *Jatti khatti* as compared to other two rootstocks. At 50 mM salinity

Table 2. Effect of salinity on photosynthetic pigments in citrus rootstocks.

Rootstock	Chlorophyll 'a' (mg g^{-1} FW)				Chlorophyll 'b' (mg g^{-1} FW)				Total chlorophyll (mg g^{-1} FW)			
Salinity →	Control	25 mM	50 mM	Mean	Control	25 mM	50 mM	Mean	Control	25 mM	50 mM	Mean
Attani-1	1.4	1.25	1.2	1.28	0.29	0.25	0.22	0.25	1.7	1.51	1.43	1.55
Attani-2	1.45	1.32	1.26	1.34	0.21	0.17	0.14	0.17	1.65	1.5	1.4	1.52
Jatti khatti	1.84	1.41	1.18	1.48	0.37	0.31	0.24	0.31	2.22	1.7	1.43	1.78
Mean	1.56	1.33	1.21		0.29	0.24	0.20		1.86	1.57	1.42	
$CD_{0.05}$												
Rootstock (R)	0.08				0.04				0.1			
Salinity (S)	0.08				0.39				0.11			
R × S	0.15				NS				0.17			

Table 3. Effect of salinity on leaf proline content in citrus rootstocks.

Rootstock	Proline content (μg g^{-1} of FW)			
Salinity →	Control	25 mM	50 mM	Mean
Attani-1	56.11	66.32	77.78	66.74
Attani-2	55.17	63.62	74.56	64.45
Jatti khatti	56.85	60.9	68.37	62.04
Mean	56.04	63.61	73.57	
$CD_{0.05}$				
Rootstock (R)		2.73		
Salinity (S)		2.70		
R × S		4.73		

level, there was 35.58% reduction over control in *Jatti khatti*, whereas it was 15.88 and 15.15% for Attani-1 and Attani-2 rootstocks, respectively (Table 2). Chlorophyll is a membrane bound pigment and its integrity depends on membrane stability. As cell membranes are damaged under saline conditions, chlorophyll seldom remains intact (Ashraf *et al.*, 1). It has been observed that reduction in chlorophyll may be due to reduced activity of specific enzymes under saline conditions (Kreps *et al.*, 11).

The data on leaf proline (Table 3) revealed that the highest (66.74 μg g^{-1} FW) content was estimated in Attani-1 plants, which was statistically non-significant as compared to Attani-2, but had significant differences as compared to *Jatti khatti*. The mean effect of salinity was also found to be significant and the highest proline content was recorded at 50 mM salinity significantly followed by 25 mM and control treatments. The rootstock and salinity interaction effect showed that rootstocks exhibited higher proline accumulation at 25 and 50 mM salinity levels as compared to the non-salinized plants. At 25 mM salinity level, the highest proline content was estimated in Attani-1 which was statistically at par with Attani-2 but significantly higher as compared to *Jatti khatti*. A similar trend was observed at 50 mM salinity. The proline accumulation increased with increasing salinity in all the rootstocks. However, the highest increase in leaf proline content was recorded in Attani-1 followed by Attani-2 and *Jatti khatti* at 50 mM salinity. It was observed that the salt-tolerant citrus rootstocks accumulate more proline as compared to the susceptible ones (Jyothi and Raijadhav, 10; Patel *et al.*, 16). Free proline increased with salinity in the leaves of lemon grafted on the relatively salt-tolerant sour orange, but not when grafted on the more salt-susceptible Alemow (Nieves *et al.*, 15). Under stress conditions, proline acts as an osmoprotectant and a storage source of nitrogen and may be engineered into citrus for higher salt tolerance.

Table 4. Effect of salinity on antioxidant enzymes activities in citrus rootstocks.

Rootstock	Superoxide dismutase (units mg^{-1}protein min^{-1})				Catalase (μ moles H_2O_2 hydrolized mg^{-1} protein min^{-1})				Peroxidase (Absorbance units g^{-1} FW)			
Salinity →	Control	25 mM	50 mM	Mean	Control	25 mM	50 mM	Mean	Control	25 mM	50 mM	Mean
Attani 1	7.48	21.35	25.00	17.94	0.92	3.96	2.03	2.30	0.19	0.39	0.27	0.28
Attani 2	8.93	26.77	36.26	23.99	1.49	5.02	3.99	3.50	0.18	0.43	0.34	0.32
Jatti khatti	11.38	31.79	45.67	29.61	1.36	10.95	3.72	5.34	0.27	0.75	0.43	0.48
Mean	9.26	26.64	35.65		1.25	6.64	3.25		0.21	0.52	0.35	
$CD_{0.05}$												
Rootstock (R)		1.71				0.58				0.06		
Salinity (S)		1.73				0.60				0.06		
R × S		2.97				1.01				0.11		

The mean effects of rootstocks and different salinity levels both were found to be significant ($P = 0.05$) with respect to SOD activity. Among different salinity levels, the highest SOD activity was recorded at 50 mM salinity. The significant differences among rootstocks revealed that the highest CAT activity was in *Jatti khatti* followed by Attani-2 and Attani-1 rootstocks. The rootstock and salinity interaction effects revealed that the highest SOD activity was in *Jatti khatti* under 50 mM NaCl salinity significantly followed by Attani-2. The mean effect of salinity revealed that the highest CAT activity was recorded at 25 mM salinity significantly followed by 50 mM and control treatments. The rootstock and salinity interaction effects showed sharp increase in salt induced CAT levels in different treatment combinations upto 25 mM salinity with the highest activity recorded in *Jatti khatti* significantly followed by Attani-2 and Attani-1 rootstocks. Contrary to the SOD, CAT activity declined in different rootstocks at 50 mM salinity (Table 4). The rootstocks differed significantly with respect to the POD activity. The highest POD activity was recorded in *Jatti khatti* significantly followed by Attani-2 and Attani-1 rootstocks. A significant difference was also observed with respect to the mean effect of salinity. Irrespective of rootstock, the highest POD activity was recorded under 25 mM salinity significantly followed by 50 mM and control. The rootstock and salinity interaction effects revealed that there was a sharp increase in salt induced POD levels in different treatment combinations upto 25 mM salinity with the highest activity recorded in the *Jatti khatti* significantly followed by Attani-2 and Attani-1 rootstocks. As with CAT, POD activity also declined in different rootstocks at 50 mM salinity. The accumulation of reactive oxygen species (ROS) such as superoxide and hydroxyl leads to the formation of lipid radicals and subsequent damage to the cell membranes. It has been reported that ROS up-regulate the antioxidant enzyme system in citrus (Wu *et al*., 18). Data presented in this study show an increase in SOD activity with increasing salt stress. However, CAT and POD activities increased upto a threshhold but declined at higher salinity level. Such different patterns of enzyme activity could be attributed to the difference in genotype, plant age, the prevailing environment and stress conditions imposed. In other crops, similar differences in enzymatic antioxidant responses between callus and intact plants have been reported (Gosset *et al*., 7).

Based on the observations, it is concluded that the relative salt tolerance among the indigenous rootstocks decreased in the following order: Attanni-1 >Attanni-2 >*Jatti khatti*.

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