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Full Length Research Paper

Exogenous ascorbic acid (vitamin C) induced anabolic changes for salt tolerance in chick pea (*Cicer arietinum* L.) plants

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The effect of exogenous supply of ascorbic acid on growth and metabolic changes in chick pea under salinity treatment was carried out in pot experiment. The exogenous supply of ascorbic acid (4 mM) improved the fresh and dry matter gain in roots, shoots and leaves of chick pea (*Cicer arietinum* L.) Plants attenuated by the salt stress (40 mM NaCl) environment in the soil solution. Significant synergistic effect between NaCl (40 mM) and ascorbic acid treatment increased the contents of chlorophyll a and chlorophyll stability index (CSI %) in leaves of chick pea plants. The total number of protein bands/lane did not change under the low (20 mM) NaCl concentration but was dramatically reduced by the high (40 mM) NaCl treatment. The sum of optical densities (O. D.) of protein bands was inhibited by both levels of NaCl, but was induced by 10.68% by the added ascorbic acid at 20 mM NaCl and by 21.39% at 40 mM NaCl. Six different polypeptides of molecular weights 146.28, 117.98, 51.55, 49.6, 44.49 and 38.34 were completely disappeared under NaCl stress (40 mM). These bands reappeared in response to the added ascorbic acid treatment. Moreover, the optical density of every individual protein band was induced by ascorbic acid under the low NaCl concentration. The results indicate synergistic interaction between salinity stress and ascorbic acid for the sake of salt resistance in chick pea plants.

Keywords: Plant growth, photosynthetic pigments, chlorophyll stability index, SDS-PAGE protein.

INTRODUCTION

Environmental stresses trigger a wide variety of plant responses, ranging from altered gene expression and cellular metabolism to accelerated leaf senescence and permanent wilting, all lead to changes in growth rates and crop yield. Salt stress adversely affects plant growth and productivity (Reddy et al., 2004; Baek et al., 2005; Amor et al., 2005), through the increase in reactive oxygen species (ROS) which may cause oxidative stress resulting in cellular damage by oxidation of lipids, proteins and nucleic acids (McKersie and Leshem, 1994; Pastori and Foyer, 2002; Apel and Hirt, 2004). To minimize the effects of oxidative salt stress, plant cells have evolved a complex antioxidant system, which is composed of low-molecular mass antioxidants as well as ROS-scavenging enzymes (Alscher et al., 1997; Apel and Hirt, 2004).

One approach for inducing oxidative stress tolerance would be to increase the cellular level of enzyme substrates such as ascorbic acid (vitamin C). Ascorbic acid is a small, water-soluble anti-oxidant molecule which acts as a primary substrate in the cyclic pathway of enzymatic detoxification of hydrogen peroxide. Improved understanding of ascorbate in plants will lead to the possibility of increasing ascorbate concentration in plants by genetic manipulation. This will have benefits for tolerance of plants to oxidative stresses (Foyer, 1993; Smirnoff, 1995).

Salinity is generally detrimental to plant growth through its adverse effects on plant metabolism that induces important modifications in gene expression. Such modifications may lead to accumulation or depletion of certain metabolites resulting in an imbalance in the levels of a relatively small set of cellular proteins, which could increase, decrease, appear or disappear after salt treatment (Kong-ngern et al, 2005).

This study investigates the effects of the exogenous supply of ascorbic acid on the growth and anabolic changes of chick pea plants with a special emphasis on the role of the cellular levels of SDS-PAGE protein profile in relation to salt tolerance.

Table 1. Growth parameters of chick pea (*Cicer arietinum* L.) plants treated with 20 mM NaCl (T1), 40 mM NaCl (T3), 20 mM NaCl + 4 mM ascorbic acid (T2) and 40 mM NaCl + 4 mM ascorbic acid (T4), respectively. C, controlled plants.

Treatment	Stem length (cm)	Root length (cm)	No. of leaves/ plant	Stem f. wt. (g)	Root f. wt. (g)	Stem d. wt. (g)	Root d. wt. (g)
C	24.33	12.50	19.67	28.67	6.72	7.33	0.433
T_1	23.67	11.00	15.33	27.91	5.67	6.63	0.336
T_2	28.67	16.33	19.00	30.19	6.44	8.33	0.322
T_3	21.75	6.67	14.67	25.80	4.33	6.11	0.288
T_4	23.33	6.83	17.33	29.67	5.11	7.21	0.367
LSD (*P* 0.05)	*5.950*	*7.515*	*4.052*	*3.055*	*1.801*	*1.476*	*0.101*

MATERIALS AND METHODS

Plant material and treatments: Pure strain of check pea *(Cicer arietinum* L.) seeds were surface-sterilized with 15% (w/v) sodium hypochlorite for 20 min, rinsed with distilled water, then soaked in plastic pots (15 cm) in pre-sieved homogenous garden soil (sandy loam). All planted pots were kept in the open garden in about 31/22ºC day/night temperature and average relative humidity 60% and irrigated regularly up to field capacity with pure water. After two weeks, planted pots were randomly subdivided into five equal groups (5 pots each). One group were kept irrigated with pure water and sampled as control. Groups 2 and 3 were subjected to 20 and 40 mM salinity (NaCl); while groups 4 and 5 received the same salinity treatment plus 4 mM ascorbic acid, respectively. Upon treatments, all pots received equal quantities of 1/2 Hoagland nutrient solution.

Growth parameters: By the end of the experiment (8 weeks), plants were smoothly uprooted, cleaned from soil residues and prepared for measurements. Plant height, root length, shoot and root fresh and dry weights, total number of leaves and number of lateral branches per plant were recorded.

Estimation of photosynthetic pigments: Chlorophyll a, chlorophyll b and carotenoids (µg/ml) contents was estimated in the fresh leaves in 80% acetone according to the procedure described by Lichtenthaler and Wellburn (1983) . Chlorophyll stability index (CSI %) was calculated by combining chlorophyll a+b contents in chick pea foliage leaves before and after salinity stress following the formula noted by Kumari et al. (2004): CSI % = Chlorophyll before stress – chlorophyll under stress / chlorophyll under stress x 100.

Protein extractions: For SDS-PAGE protein extracts were prepared by extracting appropriate weight from the frozen plant material with 0.125 M tris/ borate, pH 8.9. All the obtained extracts were kept at 4ºC for 24 h and centrifuged at 10000 rpm for 20 min. The supernatants were used for electrophoresis.

SDS-PAGE protein analysis: Exact 5 µg of each sample was separated on gel slabs according to the method of Laemmili (1970). Protein subunit bands were stained with Coomassie blue R- 250 by standard techniques. The gel was scanned using Gel pro- Analyzer ver. 3.3 (Media Cypermetics 93-97).

Statistical analysis: Data were statistically analyzed by multiple comparison procedure at p 0.05 using t-test and mean separation by least significant difference (LSD) (Steel and Torrie, 1980).

RESULTS

Plant growth: The statistical analyses of growth parameters (Table 1) revealed non significant effect on stem length, root length and stem dry weight at all salinity or salinity + ascorbic acid treatment. However, the stem and root fresh weights and the root dry weight were inhibited by the higher salinity treatment (40 mM NaCl). The number of leaves per plant was inhibited by both salinity levels (20 mM and 40 mM NaCl). The addition of ascorbic acid (4 mM) significantly increased the stem dry weight at the low level of NaCl (20 mM); while the stem fresh weight and the root dry weight were increased at the higher level of NaCl (40 mM).

Photosynthetic pigments: The spectrophotometeric estimation of chlorophyll pigments (Table 2) indicated significant inhibition of chlorophyll a content (mg/g) at the high NaCl concentration (40 mM). This inhibition was recovered by the added ascorbic acid (4 mM). The content of chlorophyll b did not show significant changes under any of the treatments. The total chlorophylls a + b was not inhibited by the applied salinity levels, but increased in response to the added ascorbic acid. On the other hand, the value of chlorophyll stability index (CSI%) was higher at the combination of 40 mM NaCl + 4 mM ascorbic acid (Figure 1).

Protein analysis: The SDS-PAGE analysis of the protein profile of chick pea plants revealed both qualitative (Figures 2 and 3) and quantitative (Table 3) changes in the banding patterns of proteins. The total number of protein bands/lane did not change under the low (20 mM) NaCl concentration but was dramatically reduced by the high (40 mM) NaCl treatment. The sum of optical densities (O. D.) was inhibited by both levels of NaCl. Six different polypeptides of molecular weights 146.28, 117.98, 51.55, 49.6, 44.49 and 38.34 were completely dis-appeared in response to NaCl (40 mM) stress. These bands reappeared in response to the added ascorbic acid treatment (4 mM) . Moreover, the optical density of every individual protein band was induced by ascorbic acid (4 mM) under the low NaCl concentration (20 mM). The sum

Table 2. Chlorophyll pigments (µg/ml) in chick (*Cicer arietinum* L.) leaves treated with 20 mM NaCl (T1), 40 mM NaCl (T3), 20 mM NaCl + 4 mM ascorbic acid (T2) and 40 mM NaCl + 4 mM ascorbic acid (T4), respectively. C, controlled plants.

Treatment	Chl a	Chl b	Chl a + Chl b	Chl a/Chl b
C	0.295	0.112	0.408	2.63
T_1	0.232	0.077	0.308	3.02
T_2	0.274	0.120	0.394	2.27
T_3	0.211	0.092	0.312	2.32
T_4	0.325	0.158	0.484	2.05
LSD (P 0.05)	0.081	0.054	0.129	0.659

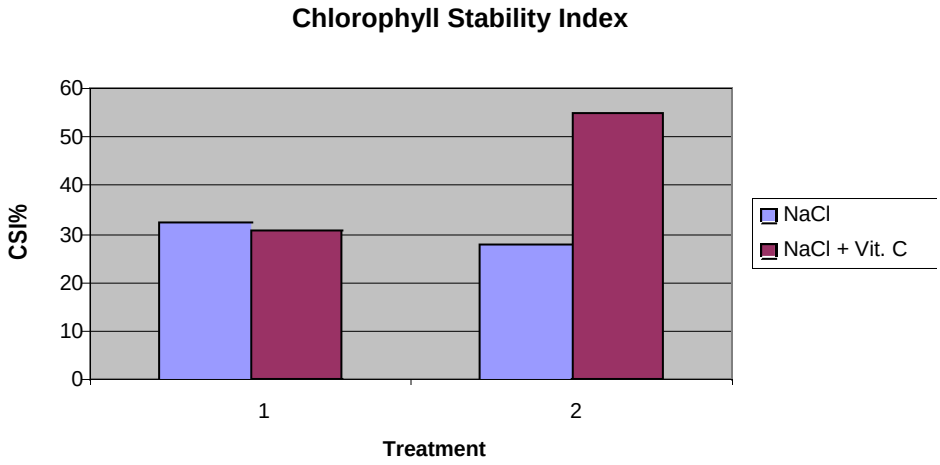

Figure 1. Chlorophyll Stability Index (CSI %) in chick pea (*Cicer arietinum* L.) plants subjected to: 1) 20 mM NaCl + 4 mM ascorbic acid and 2) 40 mM NaCl + 4 mM ascorbic acid.

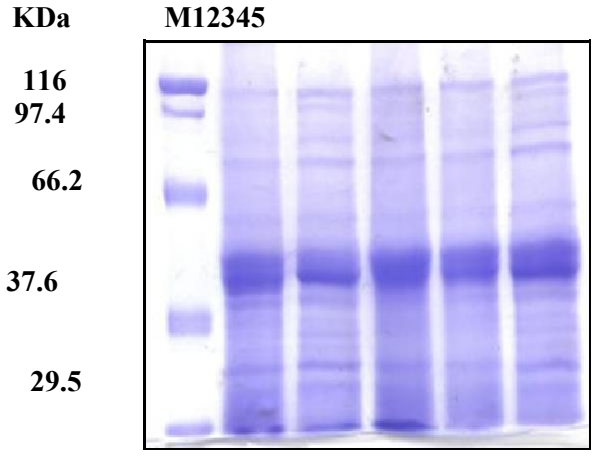

Figure 2. Electrophotograph SDS-PAGE of total proteins of chick pea (*Cicer arietinum* L.) plants. Track 1, untreated (controlled) plants; tracks 2 and 4, plants treated with 20 and 40 mM NaCl; tracks 3 and 5, plants treated with 20 mM NaCl + 4 mM ascorbic acid and 40 mM NaCl + 4 mM ascorbic acid, respectively. Track M, molecular weight markers used on polyacrylamide gel.

of O. D. was induced by 10.68% by the added ascorbic acid at the low level of salt stress and by 21.39% at the high salt concentration indicating a synergistic influence of salinity stress and ascorbic acid on salt-stressed chick pea plants.

DISCUSSION

In the present investigation, the responses of chick pea plants to high levels of salinity were reflected by decreases in stem fresh weight, root fresh weights, root dry weight and the number of leaves per plant. The stressful environment in the soil solution at a concentration of 40 mM NaCl attenuated the fresh and dry matter gain in roots, shoots and leaves. The inhibitory effects of salt stress on these parameters add more support to the ubiquitous findings of earlier investigations (Pérez-Alfocea et al., 1993; Hamada, 1996). The reduced plant growth under salt stress conditions could be attributed to the physiological drought induced by the low water potential of the soil solution and osmotic adjustments in plants as a result of increased ionic concentration in their

Table 3. Comparative analysis of optical density (O. D.), molecular weight (M.Wt.) and relative front (Rf) of SDS-PAE protein profile of chick pea (*Cicer arietinum* L.) plants treated with NaCl or NaCl + ascorbic acid.

Band number	Treatment & O. D.						Mol. Wt. (KDa)
	1	2	3	4	5	R_f	
1	65.21	49.87	80.39	48.54	57.39	0.17	521.92
2	76.01	54.80	99.86	-	67.34	0.29	146.28
3	75.43	50.11	97.14	-	55.38	0.31	117.98
4	80.81	54.51	101.01	68.75	57.56	0.38	110.25
5	90.01	74.42	108.24	74.25	69.25	0.43	104.85
6	114.62	84.83	120.90	200.05	78.21	0.55	086.37
7	227.20	197.88	231.56	157.85	208.31	0.66	066.36
8	209.61	125.07	196.29	130.35	125.92	0.73	57.00
9	156.89	102.47	-	-	99.17	0.78	51.55
10	-	-	-	-	105.44	0.80	49.60
11	141.59	137.43	-	147.37	108.83	0.85	46.40
12	186.00	207.26	207.26	-	170.24	0.88	44.49
13	-	-	-	178.55	173.23	0.93	41.13
14	233.05	189.79	249.21	-	139.56	0.97	38.34
Sum of O. D.	**1656.43**	**1328.44**	**1491.86**	**1005.71**	**1220.04**		
Bands/lane	**12**	**12**	**10**	**8**	**14**		

1 = untreated (control) plants
2 and 4 = plants treated with 20 mM NaCl and 40 mM NaCl; respectively
3 and 5 = plants treated with 20 mM NaCl + 4 mM ascorbic acid and 40 mM NaCl + 4 mM ascorbic acid; respectively.

their cells, which result in deformation of macromolecules by disrupting their shell or bound water (Schwarz, 1985).

The biochemical functions of ascorbate have been divided into four categories, (1) antioxidant, regenerates the lipophilic antioxidant -tocopherol, vitamin E (Asada, 1994); (2) enzyme cofactor for hydroxylase enzymes involved in the synthesis of hydroxyproline-rich glycol-proteins, cell wall structural proteins (Carpita and Gibeaut, 1993); (3) electron transport, acts as an in vitro electron donor and acceptor in transmembrane electron transport (Asard et al., 1995); and (4) oxalate and tartarate synthesis (Saito, 1996). The results of the current research indicated that the added ascorbic acid (4 mM) improved the stem and root fresh and dry weights of salt-stressed chick pea plants. Consistent findings reported on the beneficial effects of the exogenous application of ascorbic acid in mitigating partially the adverse effects of salt stress on growth, like cell division and cell enlargement (Mozafar and Oertli, 1992; Ahmed-Hamad and Monsaly, 1998) . Moreover, in tomato seedlings, the exogenous supply of ascorbic acid increased tissue levels (Arrigoni et al., 1997) and percentage age for surviving the toxic effects of NaCl (Shalata and Neumann, 2001). Alternative organic carbon sources without direct anti-oxidant activity did not provide equivalent protection. These remarkable protective effects appeared to be specifically related to the anti- oxidant activity of ascorbic acid, rather than its possible utility as an organic substrate for respiratory energy metabolism (Shalata and Neumann, 2001).

Ascorbate in plants occurs in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall (Anderson et al., 1983; Rauten-kranz et al., 1994) . The concentration in chloroplast can be high (up to 50 mM in spinach) and is probably related to its central role in photosynthesis (Foyer, 1993). Our results revealed significant synergistic effect between NaCl (40 mM) stress and exogenous ascorbic acid (4 mM) on the contents of chlorophyll a and chlorophyll stability index (CSI %) in the leaves of chick pea plants. Since salt stress can lead to oxidative stress through the increase in reactive oxygen species (ROS) which are highly reactive and may cause cellular damage, one of the proposed biochemical modes of ascorbate is to act as an antioxidant by scavenging hydrogen peroxide (chloroplasts lack catalase) as it forms (Miyake and Asada, 1992).

A finding of the current research is the role of the exogenous ascorbic acid in salt-stressed chick pea plants and is its ability in the induced stability of protein synthesis. The values of the band intensities (O.D.) were always higher in the low salt-stressed plants treated with ascorbic acid than in the only salt-stressed plants. In addition, the inhibited protein bands in the high salt-stressed individuals were completely restored by the added ascorbic acid. This enhanced protein stability could be partially attributed to the increased activity of antioxidant enzymes against salinity stress environment (Inzé and Van Montague, 1995; Noctor and Foyer, 1998;

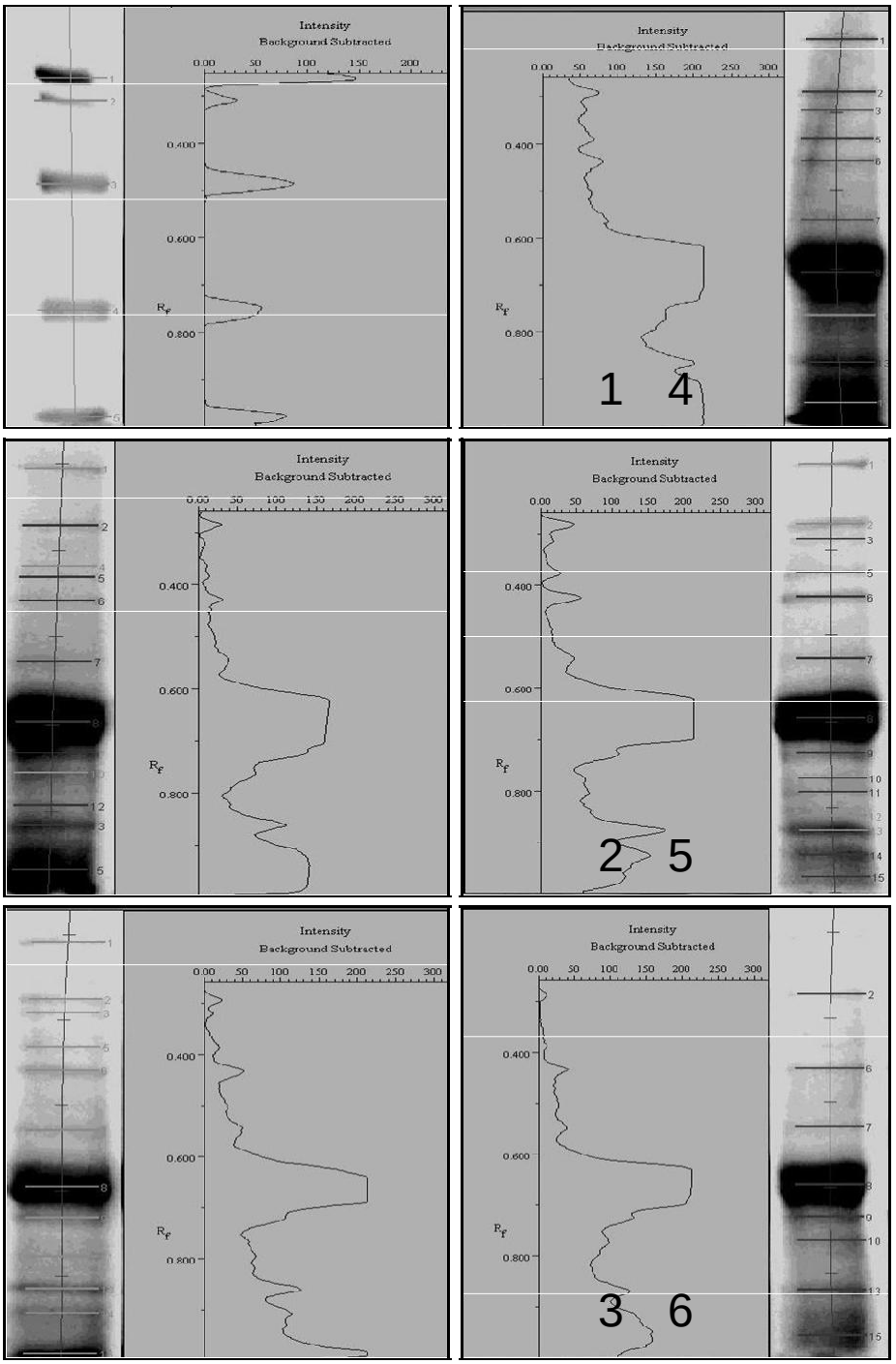

Figure 3. Scan of the tracks in the electrophotograph (Figure 2) of SDS-PAGE of total proteins of chick pea (*Cicer arietinum* L.) plants. 1, molecular weight markers used on polyacrylamide gel; 2, untreated (controlled) plants; 3 and 5, plants treated with 20 and 40 mM NaCl; 4 and 6, plants treated with 20 mM NaCl + 4 mM ascorbic acid and 40 mM NaCl + 4 mM ascorbic acid, respectively.

McKersie et al., 1999).

In conclusion, this study shows, for the first time, the remarkable synergistic interaction between the exogenous supply of ascorbic acid and the anabolic capacity of protein in salt- stressed chick pea plants. Molecular genetics studies should complement the biochemical and physiological approaches concerning of the role of ascorbate in protein expression for increased plant resistance

to salt stress.

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