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Alleviation of Salt Stress in Cucumis Sativus L. Through Seed Priming with Calcium Chloride

KEYWORDS: antioxidants; osmolyte; seed priming; salinity stress

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ABSTRACT *The present study was aimed at evaluating the potential of calcium chloride in inducing salt tolerance in Cucumis sativus L. at germination and early seedling growth stage. Response of C.sativus seeds to the pre-soaking (priming) treatment with 2 mM CaCl2 solution for 24 h was studied under saline condition (100mM and 150mM NaCl). Exogenous CaCl2 significantly ameliorated the detrimental effects of NaCl stress on germination and seedling growth. Germination potential increased by 12% in primed seeds as compared to non-primed seeds under saline condition. Shoot and root lengths of primed seedlings were two times higher than non-primed seedlings. Influence of CaCl2 priming on phenol and proline content and antioxidative enzyme activities was also studied and all these parameters were found to be significantly higher in primed seedlings as compared to non-primed seedlings. These findings suggested that the beneficial effects of CaCl2 priming on germination and seedling growth could be due to its role in activation of antioxidant system and accumulation of proline.*

Introduction

Salinity is one of the major abiotic stresses hampering crop productivity worldwide. It is well known that soil salinity adversely affects germination percentage and early seedling growth (Jamil et al. 2006). However, priming of seeds with water (Casenave and Toseli 2007), inorganic salts (Patade et al. 2009), osmolytes (Iqbal and Asraf 2007) and hormones (Iqbal and Asraf 2007) has been demonstrated as a successful cost-effective strategy for improving seed vigour and seedling growth under saline conditions (Foti et al. 2008).

It is known that salinity interacts with plant nutrients (Hu and Schmidhalter 2005). For instance, Ca2+ and K+ are decreased in plants under saline conditions (Hu and Schmidhalter 2005).Under stress, plants accumulate or release intracellular cytosolic calcium, which acts as a signal and regulate a range of physiological processes to combat the stress (Kader and Lindberg 2008). It provides protection against sodium toxicity through several mechanisms including increasing the selectivity of root K+/Na+ uptake (Bolat et al. 2006), reducing membrane damage and electrolyte leakage thereby providing membrane stability (Bolat et al. 2006), readjustment of carbon metabolisn (Anil et al. 2005), improving water transport in plants by regulating aquaporin activity (Carvajal et al. 2000; Cabanero et al. 2006).

Many workers have reported calcium chloride priming induced tolerance in plants in terms of improved germination and stand establishment under saline conditions (Rafiq et al. 2006, Afzal et al. 2008). However, the underlying mechanisms associated with tolerance are not fully understood. Study of influence of CaCl2 priming on key biochemical and physiological processes affecting plants' growth under saline conditions may provide insight into its role in regulating these processes.

The present investigation was undertaken to evaluate the potential of calcium chloride in alleviating salt–induced alterations in early seedling growth in *Cucumis sativus* L., a crop known for its high salt sensitivity (Zu et al. 2008). It was also aimed to draw relationship between growth and biochemical processes associated with $CaCl_2$ priming induced salinity tolerance.

Materials and Methods

2.1 Seed germination and seedling growth

Seeds of *Cucumis sativus* L. variety Himani obtained from Ratanshi Agro-Hortitec, Byculla, Mumbai, India were used for the present study.

The concentration of priming agent ($CaCl_2$) was first optimized so as to select the optimal dose. Among the different concentrations (1.0 mM, 2.0mM, 3.0 mM, 4.0 mM) tested, seeds treated with 2mM $CaCl_2$ showed maximum germination (data not shown) and hence the same concentration was used for the present study.

Seeds were surface sterilized for 3 min. with 0.1% $HgCl_2$, and rinsed with sterile distilled water. For priming, seeds were soaked in 2mM calcium chloride solution for 24 hours. Both primed and non-primed (soaked in sterile distilled water for one hour) seeds were subsequently placed in 9 cm Petri dishes lined with Whatman no.1 filter paper moistened with solutions of two NaCl concentrations (100mM and 150 mM). For comparison, a part of non-primed seeds were placed in petri plates lined with filter paper moistened with distilled water (control). The Petri dishes were sealed with parafilm "M" to prevent drying and placed in a growth chamber at 28±2°C, with a photoperiod of 16 h providing 45 µmole.m^{-2}·s^{-1} illumination provided by cool white fluorescent tubes. The experiments were performed twice and three replicates of thirty seeds each were used for all the treatments.

Number of days required for initiation of seed germination was recorded as Mean germination Time calculated according to the equation of Ellis and Roberts (1981):

$$MT = \frac{\sum Dn}{\sum n}$$

where: *n* is the number of seeds, which were germinated on day *D*, and *D* is the number of days counted from the beginning of germination.

A seed was considered to have germinated when the emerging radical elongated to 1 mm. Germination potential was recorded every 2 days for a period of 15 days. Seedling root

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and shoot lengths were measured after 15 days of sowing and were subsequently used for biochemical analysis.

2.2 Chlorophyll Content
For chlorophyll extraction, fresh seedlings was ground in 80% acetone and the absorbance was read spectrophotometrically at 663 and 645 nm. Total chlorophyll content ($mg.g^{-1}$fresh weight) was estimated by the method of Arnon (1949).

2.3 Proline Content
Proline content was estimated following the procedure of Bates et al. (1973). Seedlings (ca. 500 mg) were homogenized in 5.0 ml ice cold 3% aqueous sulphosalicylic acid in an ice cold mortar and pestle. The homogenate was centrifuged at 15, 652 g for 15 min. at 4°C. To an aliquot of 2 ml supernatant, 2 ml of acid ninhydrin was added followed by addition of 2 ml of glacial acetic acid, boiled for 1 hour and allowed to cool. The reaction mixture was extracted with toluene (4.0 ml), vortexed and free proline estimated from the organic phase by recording absorbance at 520 nm using a UV-Visible spectrophotometer. A 1 $mg.ml^{-1}$ solution of proline, as a standard, was used to calculate proline concentration in the samples.

2.4 Total Phenols
Total phenolic content was determined by the Folin-Ciocalteu method (Singleton et al. 1999). A 0.5ml of methanolic extract of seedlings was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water, followed by 2.5 ml of 7.5% $NaHCO_3$ solution. The mixture was incubated in a shaking incubator at 45° C for 45 min and its absorbance at 765 nm was measured. Gallic acid was used as standard for preparation of calibration curve. Total phenolic contents were expressed in terms of gallic acid equivalent as gallic acid (mg gallic acid g^{-1} fresh weight).

2.5 Hydrogen peroxide scavenging activity
The H_2O_2 scavenging activity was determined by replacement titration (Zhang, 2000). Seedlings (0.5g) were homogenized in 1.5 ml 0.1 M phosphate buffer in mortar and pestle. The homogenate was centrifuged at 15, 652 g for 15 min. at 4°C. Aliquot of 1.0 ml of 0.1mM H_2O_2 and 1.0 ml of various concentrations of supernatant were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml 2M H_2SO_4 and 7.0 ml 1.8M potassium iodide. The solution was mixed and titrated against 5.09 mM sodium thiosulphate (NaS_2O_3), until yellow colour disappeared. Percentage of scavenging of H_2O_2 was calculated as:

% inhibition = (V0-V1)/V0 x100

Where, V0 was the volume of NaS_2O_3 solution used to titrate the control (without plant extract) in the presence of H_2O_2 and V1 was the volume of NaS_2O_3 solution used in the presence of plant extracts.

2.6 Antioxidative enzyme activities
2.6.1 Protein Extraction

Seedlings (ca 500 mg) from the control and each treatment (different concentrations of NaCl) were homogenized using a chilled mortar and pestle in (tissue/buffer ratio 1:2, w/v) ice cold extraction buffer, pH 8.0, containing 0.1 M phosphate buffer and 1% polyvinylpoly pyrolidine (w/v). The homogenate was centrifuged at 13,300 g for 15 min at 4°C. The resultant supernatant was used for determination of catalase and peroxidase activity.

2.6.2 Catalase (CAT) activity
Catalase activity was determined by the method of Braber (1980). To 300 µL of solution containing 0.1 M phosphate buffer (pH 7.0) and 200 µL 0.005M hydrogen peroxide, 100 µL of protein extract was added and left at 25°C for 5 min. Then 1 ml of sulphuric acid (0.7 N) was added and the reaction mixture titrated with potassium permanganate (0.01 N) until a pink color was achieved and persisted for at least 15 sec. The enzyme activity was expressed as µM of H_2O_2 used min^{-1} g^{-1} fresh weight.

2.6.3 Peroxidase (POD)activity
Peroxidase activity was carried out according to Miranda et al. (1995).The reaction mixture contained in 1ml: 8mM H_2O_2, 40mM guaiacol, 50mM sodium acetate buffer, pH 5.5 and least amount of enzyme preparation. The change in absorbance at 470 nm due to guaiacol oxidation was followed for 1min using a spectrophotometer. One unit of peroxidase activity was defined as the amount of enzyme which increases the OD 1.0 per min under standard assay conditions.

2.7 Statistical Analysis
The experiment was arranged in a completely randomized design with three replications. Data recorded in each replication were pooled and subjected to analysis of variance (ANOVA) using SPSS (ver. 14, SPSS Inc., Irvine, Calif.). Treatment means were separated with LSD test at 5% level of probability.

Results and Discussion
Priming of seeds with inorganic salts (halo priming) is a very common and effective way to overcome the salinity problem in agricultural lands. The present study demonstrated the potential of $CaCl_2$ in alleviating salinity stress in *C. sativus* L. Analysis of variance revealed that all the growth and biochemical parameters examined were significantly ($p < 0.05$) affected by calcium chloride priming. Emergence was delayed (9 days) under saline conditions as compared to control (3 days) and was improved (5 days) upon seed priming with 2 mM $CaCl_2$ under both the saline conditions (100mM and 150 mM).

Germination Potential
Seed germination is the most important stage in plant establishment under saline condition. It is generally inhibited under salinity (Rafiq et al. 2006) which is attributed to osmotic stress resulting in decreased water absorption by the seeds. In the present study germination declined by 23% and 31% at 100 mM and 150 mM NaCl concentrations, respectively, compared to control. Priming of seeds with $CaCl_2$ improved the germination potential by 12% and 8% at 100mM and 150 mM NaCl, respectively, as compared to non-primed seeds (Table 1). According to Mozafari et al. (2008) calcium is required to maintain structural integrity of α-amylase, a key enzyme required for germination. Similar to our observations, enhanced germination potential upon $CaCl_2$ priming has also been reported in maize (Afzal et al. 2008) and Wheat (Ashraf and Rauf 2001) and this could be due to calcium mediated improved stability and activity of α-amylase (Saboury and Karbassi, 2000).

3.2 Seedling growth
In the present study, seedling shoot and root length significantly declined under NaCl stress conditions as compared to control. The decline however, was more pronounced for root length with more than 50 % inhibition under both the NaCl levels (Table 1). Growth reduction in plants exposed to salinity is associated with NaCl toxicity and unbalanced nutrient uptake (Jamil et al. 2006). Calcium application restricts the intake of Na^+ thereby protecting from its toxic effects (Anil et al. 2005) and leads to improved growth. This reason supports our observations on $CaCl_2$ mediated stimulation of growth of stressed seedlings. The shoot length was 2.5 times higher whereas the root length was two times higher as compared to non-primed seeds under both the NaCl levels (Table 1). This was consistent with the higher values of seedling growth parameters in cucumber reported by Ghassemi and Esmaeilpour (2008).

3.4 Total Chlorophyll Content
Salinity leads to an increase in free radicals in chloroplast and this causes destruction of chlorophyll molecules by them re-

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sulting in reduced photosynthesis and growth (Lichtenthlaler et al. 2005). In the present study, salt stress reduced the total chlorophyll content of cucumber seedlings by 50 % at both the NaCl levels and this could be associated with seedling growth inhibition observed under salinity stress.

As compared to non-primed seedlings, priming of seeds with $CaCl_2$ doubled the chlorophyll content under 100 mM NaCl (Table 1) whereas at 150 mM NaCl there was no significant increase. According to Montesano and Van Iersel (2007) calcium prevents the toxic effects of NaCl on photosynthesis and this appeared to be the reason for priming induced improved chlorophyll content of seedlings of *C. sativus* observed in our study, which is in agreement with Afzal et al. (2012). Yeo et al. (1991) observed that chlorophyll membrane structure was protected and photosynthetic proteins were activated in calcium treated plants and such plants grew well. Priming- induced improved growth of stressed seedlings observed in the present study was found to be associated with their enhanced chlorophyll content.

After observing the positive effects of priming on germination and seedling growth, the next objective was to assess biochemical responses in an effort to identify process/es responsible for improved survival and vigour under salinity. For this purpose, effect of $CaCl_2$ priming on osmolyte content and antioxidative system of seedlings grown under saline conditions was evaluated.

3.5 Proline content

Osmolytes accumulation has been frequently reported in plants exposed to salinity and is generally correlated with plant's ability to tolerate and adapt to saline conditions (Errabii et al. 2007). Proline content of seedlings grown under control condition was 3.96 $\mu g.g^{-1}$ FW. Significant increase in its accumulation was recorded in seedlings exposed to NaCl stress. It was 5.57 $\mu g.g^{-1}$ FW and 7.06 $\mu g.g^{-1}$ FW at 100 mM and 150 mM NaCl concentration, respectively. In the present study improved survival and seedling growth at 100 mM NaCl could be associated with better osmotic adjustment as indicated by high proline accumulation in primed seeds as compared to non-primed seeds (Table 2). Enhanced proline content upon calcium chloride priming under saline conditions has also been observed in rice (Chaum et al. 2012), linseed (Khan et al. 2010) and bean (Cabot et al. 2009). According to Girija et al. (2002) proline accumulation could probably be due to breakdown of proline rich protein or *de novo* synthesis of proline.

3.6 H_2O_2 Scavenging activity, peroxidase and catalase activity

Besides osmotic stress another reason for salinity induced damage is generation of reactive oxygen species (ROS) (Zu 2001). In particular, it is known that H_2O_2 is a strong inhibitor of calvin cycle, and for this reason, it must be eliminated by conversion to H_2O in reactions involving POD and CAT. In the present study H_2O_2 scavenging activity of NaCl-stressed seedlings was higher (8% and 11% at 100mM and 150mM NaCl, respectively) than control and was further significantly enhanced by $CaCl_2$ priming (Table 2). The same trend was observed for the activities of CAT and POD. Peroxidase activity was 14% and 28% higher (Table 2) and catalase activity was 25% and 43% higher (Table 2) at 100 mM and 150 mM NaCl, respectively as compared to control. Priming of seeds with $CaCl_2$ further increased the activity of both enzymes as compared to non-primed seedlings under both the NaCl levels. The increase however, was more significant under 100mM NaCl (15%for POD and 32% for CAT) as compared to 150 mM NaCl (6% for POD and 20% for CAT).

Interestingly, priming-induced enhanced CAT and POD activity observed in the stressed seedlings in our study corresponded to increased H_2O_2 scavenging activity, indicating neutralization of H_2O_2 by CAT and POD. Calcium treatment induced antioxidant system has also been reported in salt stressed Jerusalem artichoke (Xue et al. 2008) and linseed (Khan et al. 2010).).

3.7 Total Phenols

In addition to antioxidative enzymes, antioxidants such as phenols, being equipped with the ability to act as hydrogen donators, reducing agents and quenchers of singlet oxygen, also have an important role in abiotic stress tolerance (Hanen et al. 2008). Phenol content of stressed seedlings was two times higher than control at 100 mM NaCl and priming of seeds with calcium chloride further increased the phenol content by 24% over non-primed seedlings (Table 2). Our results on enhanced phenol contents of primed seedlings suggested this to be an adaptive mechanism for scavenging ROS, thereby mitigating oxidative stress and improving growth under salinity stress. Jafar et al. (2012) also observed increased phenols in saline conditions upon halopriming in wheat and correlated it with stimulated growth.

In conclusion, $CaCl_2$ priming was effective in alleviating salt stress effects on germination and early seedling growth in *C. sativus* L. Our data suggested that priming-induced acquisition of salinity tolerance in *C. sativus* L. could possibly be due to activation of antioxidant system and accumulation of proline.

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Table 1: Effect of $CaCl_2$ priming on biochemical parameters of *Cucumis sativus* L. seedlings grown under NaCl stress.

Treatments	Proline Content ($\mu g.\ g^{-1}$ fw)	Peroxidase activity ($U.h^{-1}\ g^{-1}$ fw)	Catalase activity ($\mu M\ g^{-1}$fw min^{-1})	H_2O_2 scavenging activity(% Inhibition)	Total phenols (mg. g^{-1} fw)
Distilled water (control)	3.96[d]	86.99[e]	671.66[e]	50.00[d]	2.24[c]
NaCl 100mM	5.57[c]	101.66[d]	899.76[d]	58.38[c]	3.24[c]
2mM $CaCl_2$+NaCl100mM	6.80[b]	118.00[c]	1340.33[b]	63.23[bc]	4.30[b]
NaCl 150mM	7.06[a]	122.1[b]	1196.66[c]	61.54[c]	4.50[ab]
2mM $CaCl_2$+ $NaCl^2$ 150mM	7.33[a]	130.0[a]	1496.70[a]	68.30[a]	4.86[a]

Means followed by different letters differ significantly at 5%level of probability, LDS.

Figure Legends

Fig.1 Effect of $CaCl_2$ priming on (a) Germination potential, (b) Shoot length, (c) Root length and (d) Total chlorophyll content of *Cucumis sativus* L. under salinity stress. The bars with differen letters differ significantly at P<0.05, LSD Test).

Fig. 2 Effect of $CaCl_2$ priming on (a) Proline content, (b) H_2O_2 Scavenging Activity, (c) Total phenols, (d) Peroxidase activity and (e) Catalase activity of *Cucumis sativus* L.under salinity stress. The bars with different letters differ significantly at P<0.05, LSD Test).

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