

Full Length Research Paper

Effects of calcium paste as a seed coat on growth, yield and enzymatic activities in NaCl stressed-pea plants

Saad M. Howladar¹ and Mostafa M. Rady^{2*}

¹Biology Department, Faculty of Sciences, Al-Baha University, Al-Baha, Saudi Arabia.

²Botany Department, Faculty of Agriculture, Fayoum University, 63514-Fayoum, Egypt.

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The present study was conducted to study the effect of coating the seeds with calcium paste before sowing, on plant growth, yield, the contents of some antioxidants and the activities of carbonic anhydrase and nitrate reductase in the *Pisum sativum* L. leaves under the influence of NaCl stress. NaCl stress reduced plant growth, photosynthetic pigment levels, ascorbic acid and calcium contents, and the activities of carbonic anhydrase and nitrate reductase. In contrast, proline and sodium contents were increased. These results are negatively reflected in the yield components. However, seed coating with calcium paste reduced the toxic effects of NaCl on plant growth and yield by increasing leaf pigments, ascorbic acid, proline contents and enzymatic activities. This study clearly highlights the effects of calcium paste as a seed coat in mitigating the phytotoxicity of NaCl stress in pea plants.

Key words: Calcium paste, carbonic anhydrase, nitrate reductase, ascorbate, growth, yield, *Pisum sativum* L.

INTRODUCTION

Pea (*Pisum sativum* L.) is one of the most popular vegetable crops in Middle East. It is considered as one of the main leguminous crops that is an important component of the agricultural sector in developing countries due to its ability to produce significant quantities of protein, carbohydrates and nutrient-rich seed. Pea is widely cultivated on newly-reclaimed soils in Middle East. However, most newly-reclaimed soils are affected by salinity and have low fertility and a poor soil structure. Saline conditions disrupt several physiological processes in plants leading to a general reduction in growth and yield (Munns, 2002; Howladar, 2010; Rady, 2011). The drastic influence of salinity on plant growth and metabolism attributes, principally, to the enhanced Na⁺ uptake, thus causes ion excess in plant tissues. One of the primary effects of increasing salinity in the growth medium is the inhibition of K⁺, Ca²⁺ and NO₃⁻ uptake by plant roots (Abbas et al., 1991). In contrast, the increase

in Na⁺ and Cl⁻ levels in plant shoots occurs with the increase in salt concentration (El-Hendawy et al., 2005). In addition, it is well established that salinity stress damages plant cell through production of reactive oxygen species including superoxide, hydrogen peroxide, hydroxyl anions and singlet oxygen (Hameed et al., 2008). Efforts have been made to control salinity by technological means; reclamation, drainage, use of high leaching fractions and application of soil amendments (Abdel-Naby et al., 2001). In recent years, much attention has been paid on the development of sustainable agriculture; hence, natural materials have been applied as soil amendments to overcome the adverse effects of soil salinity, to improve the physical and chemical properties of soils, to increase their water retention, and as fertilizers during the plant growth period.

The application of humic acid, as an organic soil application used either individually or in combination with others, resulted in a significant increase in plant growth, and crop yield and its components in the sandy soil through its improvement of hydrophysical properties and nutrient availability (Osman and Ewees, 2008). Humic acids enable growing plants to overcome the adverse effects of moderate soil salinity.

*Corresponding author. E-mail: mrady2009@yahoo.com. Tel: 00201007302668 or 00201119092061. Fax: 002846343970 or 002846334964.

Table 1. Major components of the two types of calcium paste (CW and CWH) used in this experiment.

Major component	CW	CWH
Ach [% (w/w)]	7.36	6.64
Ca ²⁺ [% (w/w)]	5.63	4.76
Humic acid [% (w/w)]	-	7.75
Total fibers [% (w/w)]	36.67	32.26
Water holding capacity (g/g)	4.70	5.80

Calcium is considered as an important factor in maintenance of membrane integrity and ion-transport regulation. It is essential for K⁺/Na⁺ selectivity and membrane integrity (Hanson, 1984). Elevated Ca²⁺ concentrations in the nutrient solution mitigated the adverse effects of salinity by inhibition of Na⁺ uptake and by reduction in leakage of membranes (Hepler, 2005). He added that the Ca²⁺/Na⁺ interactions take place at the plasmalemma. They suggested that Na⁺ acted by displacing Ca²⁺ from membranes, leading to increased membrane permeability and intracellular Na⁺ concentrations.

Due to considerable evidence of the adverse effects of soil salinity on plant growth, it was hypothesized that the different types of calcium paste used in this study as a seed protecting coat can assuage the injurious effects of 150 mM NaCl stress on pea plants. Thus, the primary objective of this work was to examine whether or not the calcium paste could mitigate the inhibiting effects of NaCl stress and regulate plant growth by adjusting the ascorbate and proline content, and the activities of some enzymes involved in stress tolerance.

MATERIALS AND METHODS

Plant material and growth conditions

The seeds of pea (*Pisum sativum* L. cv. Master-B, the pea cultivar most sensitive to salinity) were obtained from the Agricultural Research Center (Department of Vegetable Crops, Giza, Egypt). Two types of calcium paste [calcium sulphate + wheat bran (CW) or calcium sulphate + wheat bran + humic acid (CWH)] were used in this experiment. CW consisted of calcium sulphate + wheat bran (a by-product of wheat grain grinding) at a ratio of 1:5 (w/w), respectively, CWH consisted of calcium sulphate + wheat bran + humic acid (Alpha Chemika, Mumbai, India) at a ratio of 2:10:1 (w/w/w), respectively. To obtain the calcium paste, the components were mixed and kneaded together using Arabic Gum solution (5%) as a sticking agent. Except for seeds of the control and 150 mM NaCl treatments, the healthy seeds were well coated with calcium paste by manual stirring. Each 1 kg seed needed 500 g of CW or CWH for well coating and to be available to seeds and roots in a longer time during vegetative growth stage. CW- or CWH-coated seeds were then spread on a plastic sheet and allowed to dry overnight under room temperature (Table 1).

Three pre-treated or untreated seeds were sown on 15 November 2010 and 2011, in each plastic pot (40 cm in diameter, 50 cm in deep) filled with acid then deionized water washed sand. Plants were irrigated with ½-strength Hoagland solutions every

three days throughout the duration of the experiment. Plants were grown in an open-roof greenhouse. The average day and night temperatures were 20 ± 3 and 12 ± 2°C, respectively. The relative humidity ranged from 60.4 to 65.2%, and day-length from 11 to 12 h. After our preliminary studies, NaCl at the 150 mM was found to be the most significant in inhibiting growth of pea plants (data not shown). Therefore, 150 mM of NaCl was used, once every six days until the 40th day in which the CW or CWH completely disappeared from rhizosphere, along with the nutrient solution to study the effect of seed coating with calcium paste on salinity phytotoxicity resistance, especially during the vegetative growth period. Each treatment consisted of 20 pots/replicates. Samples were collected at 40 days after sowing to assess growth parameters, leaf pigments, ascorbate, proline, Ca²⁺, Na⁺ and relative water contents, and the activities of some enzymes. The yield components were determined at the end of experiment (at green pod harvest).

Plant growth and yield analyses

The plants were removed from the pots above the sand surface and the shoot lengths were measured by using a meter scale. After weighing the shoots for fresh weight, they were placed in an oven at 70°C for 48 h. Dried shoots were weighed to record the dry weight. The leaf area was measured manually by using a graph sheet, where the squares covered by the leaf were counted to note the leaf area. At the end of the experiment, green pods were collected and weighed. Then, green seeds were extracted from pods and weighed.

Determination of pigment content

Total chlorophyll and carotenoids contents (mg g⁻¹ FW) were estimated by adopting the procedure given by Arnon (1949). Leaf discs (2 cm in diameter) were homogenized with 80% acetone and centrifuged; the optical density of the acetone extract was measured at 663, 645 and 470 nm using an ultra violet (UV)-160A UV visible recording spectrometer, Shimadzu, Japan.

Determination of ascorbic acid, relative water and proline contents

The ascorbic acid content (mg 100 g⁻¹ FW) were determined using the 2,6-dichloro-indophenol method (Helrich, 1990). Frozen leaf samples were pulverised in a domestic grinder (Magefesa, Spain) and triplicate 10 g aliquots of each sample were immediately homogenized in 50 ml (w/v) of metaphosphoric acid/acetic acid solution.

The extracts were centrifuged for 15 min at 7,000 ×g, filtered through six layers of cheese-cloth, and made up to 100 ml (v/v) with

Table 2. Effect of seed application with calcium paste on the plant growth in terms of shoot length (SL; cm), number of branches plant⁻¹ (NB), leaf area plant⁻¹ (LA; cm²), shoot fresh weight plant⁻¹ (SFW; g) and shoot dry weight plant⁻¹ (SDW; g) in pea plants grown under salinity stress (n = 10).

Treatment	Parameter				
	SL	NB	LA	SFW	SDW
Control	21.7 ^b	1.25 ^d	216.8 ^c	7.98 ^c	4.18 ^c
CW*	24.1 ^a	1.50 ^c	265.1 ^b	8.67 ^b	5.79 ^b
CWH*	28.1 ^a	2.00 ^a	299.2 ^a	9.32 ^a	7.72 ^a
NaCl (150 mM)	12.3 ^d	1.00 ^e	101.1 ^c	4.16 ^f	2.57 ^e
NaCl + CW	18.4 ^c	1.50 ^c	206.7 ^c	6.59 ^e	3.54 ^d
NaCl + CWH	21.8 ^b	1.75 ^b	270.0 ^b	7.27 ^d	4.20 ^c

*CW, Calcium paste consists of CaSO₄ + wheat bran at the ratio 1:5 (w/w). *CWH, Calcium paste consists of CaSO₄ + wheat bran + humic acid at the ratio 2:10:1 (w/w/w).

metaphosphoric acid/acetic acid solution. Triplicate aliquots of each sample were titrated with 2,6-dichloro-indophenol solution. Ascorbic acid reduces the 2,6-dichloro-indophenol to a colorless solution and a slight excess of unreduced dye resulting in a characteristic light-pink color, indicating the end point of the reaction (Helrich, 1990).

The relative water content (RWC) was determined in fresh leaf discs of 2 cm² diameter, excluding midrib. Discs were weighed quickly and immediately floated on double distilled water (DDW) in Petri dishes to saturate them with water for the next 24 h, in dark. The adhering water of the discs was blotted and turgor mass was noted. Dry mass of the discs was recorded after dehydrating them at 70°C for 48 h. RWC was calculated by placing the values in the following formula:

$$\text{RWC} = \frac{[(\text{fresh mass} - \text{dry mass}) / (\text{turgor mass} - \text{dry mass})] \times 100}{\text{Hayat et al., 2007}}$$

The proline content (mg g⁻¹ DW) in leaf samples was measured by rapid colorimetric method of Bates et al. (1973). Proline was extracted from 0.5 g of dried seedling samples by grinding in 10 ml of 3% sulphosalicylic acid. The mixture was then centrifuged at 10,000 xg for 10 min. Two milliliter of the supernatant was added into test tubes and 2 ml of freshly prepared acid-ninhydrin solution was added also. Tubes were incubated in a water bath at 90°C for 30 min. The reaction was terminated in ice-bath. The reaction mixture was extracted with 5 ml of toluene and vortexed for 15 s. The tubes were allowed to stand at least for 20 min in darkness at room temperature to allow the separation of toluene and aqueous phases. The toluene phase was then carefully collected into test tubes and toluene fraction was read at 520 nm. The proline concentration in the sample was determined from a standard curve using analytical grade proline and then calculated on dry weight basis.

Determination of calcium (Ca) and sodium (Na) contents

Leaf Ca and Na contents (mg g⁻¹ DW) were assessed. Ca was determined using a Perkin Elmer Model 3300 Atomic Absorption Spectrophotometer (Chapman and Pratt, 1961). Na was determined using a Perkin Elmer Model 52-A Flame Photometer (Page et al., 1982).

Determination of enzymes activities

Carbonic anhydrase (CA, E.C. 4.2.1.1) was assayed according to Dwivedi and Randhawa (1974). Fresh leaf samples (200 mg) were cut into small pieces in 0.2 M cysteine hydrochloride solution.

These pieces were transferred to test tubes containing phosphate buffer (pH 6.8). Solutions of sodium bicarbonate (0.2 M) and bromothymol blue were added to the reaction mixture. Carbon dioxide (CO₂) liberated during catalytic action of CA on NaHCO₃ was estimated by titrating the reaction mixture against 0.05 N HCl using methyl red as an indicator.

Nitrate reductase activity (NR, E.C. 1.6.6.1) was determined in fresh leaf samples using the procedure described by Jaworski (1971). This method is based on the reduction of nitrate to nitrite, whose values were estimated calorimetrically.

Statistical analysis

The values for the parameters were subjected to statistical analysis, following the standard procedure described by Gomez and Gomez (1984). The 'F' test was applied to assess the significance of the treatment, at 5% level of probability.

RESULTS AND DISCUSSION

NaCl substantially reduced the growth of pea plants as compared to that of unstressed plants (Table 2). However, supplementation of NaCl with calcium paste (CW or CWH) considerably removed the inhibitory effect of NaCl. CWH was found to be more effective in alleviating the inhibitory effect of NaCl stress. It improved the growth of pea in terms of shoot length, number of branches plant⁻¹, leaf area plant⁻¹, shoot fresh weight and shoot dry weight by 77, 75, 167, 75 and 63%, respectively, over stressed control. The application of calcium paste alone also had a significant effect and increased all the growth parameters by 29, 60, 38, 17 and 85%, respectively, over unstressed control. The ability of pea plants to tolerate toxic levels of NaCl was found to increase due to the seed application with calcium paste (CW or CWH). The positive results of growth, which were restored to the level of unstressed control, were obtained with the calcium paste as a result, overcoming the harmful effects of soil salinity during the vegetative growth duration by calcium paste components; Ca²⁺ as an antagonist for excluding Na⁺ from rhizosphere, humic acid as a soil amendment to improve

Table 3. Effect of seed application with calcium paste on the total leaf chlorophyll (T. chl.), carotenoids (T. carot.) and ascorbic acid (ascorbate) contents (mg g^{-1} fresh weight) as well as on relative water content (RWC; %) and proline content (mg g^{-1} dry weight) in pea plants grown under salinity stress ($n = 10$).

Treatment	Parameter				
	T. chl.	T. carot.	ascorbate	RWC	proline
Control	1.65 ^b	0.38 ^c	1.04 ^c	74 ^c	0.063 ^e
CW*	1.70 ^b	0.45 ^b	1.17 ^b	80 ^b	0.071 ^e
CWH*	1.97 ^a	0.52 ^a	1.31 ^a	87 ^a	0.080 ^d
NaCl (150 mM)	0.83 ^d	0.22 ^e	0.90 ^d	48 ^e	0.094 ^c
NaCl + CW	1.46 ^c	0.32 ^d	1.03 ^c	66 ^d	0.107 ^b
NaCl + CWH	1.78 ^b	0.40 ^{bc}	1.22 ^{ab}	74 ^c	0.122 ^a

*CW, Calcium paste consists of CaSO_4 + wheat bran at the ratio 1:5 (w/w). *CWH, Calcium paste consists of CaSO_4 + wheat bran + humic acid at the ratio 2:10:1 (w/w/w).

the soil characteristics which was defected due to salinity. In addition, wheat bran acted as a water holding material in the rhizosphere to cope with drought, resulting in soil salinity.

Plants treated with NaCl showed a decrease in total chlorophyll, carotenoids and ascorbic acid contents by 50, 42 and 13%, and revealed an increase in free proline content by 27% as compared to the control, that is, unstressed plants (Table 3). However, treatment of pea plants under stress with calcium paste mitigated the inhibitory effect of salt stress and restored the pigment level in plants. CWH was found to be highly effective in increasing the total chlorophyll, carotenoids, ascorbic acid and free proline contents by 114, 82, 36 and 30%, respectively, as compared to stressed control, and by 8, 5, 17 and 94%, respectively, as compared to unstressed control. The results of the present study showed that pretreatment of seeds with calcium paste enhanced the synthesis of photosynthetic pigments and resulted in restoration of antioxidant level in the form of ascorbic acid in leaves of pea plants challenged with NaCl stress. Ascorbic acid is known to operate as an antioxidant either in direct chemical interaction with free oxyradicals or during the reaction catalyzed by ascorbate peroxidase (Nakano and Asada, 1981). The accumulation of ascorbic acid led to an enhancement of plant tolerance toward salinity stress. The mechanism by which humic acid (a component of calcium paste) stimulates plant growth may be similar to that of plant growth regulators. Humic substances include auxins, or function as auxins, and thus affect plant metabolism in a positive manner (Osman and Ewees, 2008). This may explain the positive influence of calcium paste on proline and chlorophyll contents under saline soil conditions, which was then positively reflected in the growth of pea plants. Humic acids causes higher rates of uptake of elemental K and therefore a corresponding increase in chlorophyll fluorescence which can serve as an indicator of stress induced by alterations in the balance of endogenous hormones (Marschner, 1995). Furthermore, Ca^{2+} acts as

antagonizer for distant Na^+ from the rhizosphere, in addition to diluting the concentration of salts in soil solution which may occurs by wheat bran as a water holding material. The increase in ascorbic acid in pea plants occurred as a result, seed application with calcium paste in this study (Table 3) was found to be one of plant antioxidants that enabled plants to overcome the adverse effects of NaCl stress.

Data presented in Table 3 shows the response of RWC in pea plants treated with NaCl and calcium paste application. NaCl treatment significantly decreased RWC and the loss was in proportion to the concentration of the NaCl. However, calcium paste treatment elevated RWC in NaCl-stressed plants. RWC in leaves is considered as a measure of plant water status, reflecting the metabolic activity in plant tissues (Flower and Ludlow, 1986). The ability of wheat bran in water retention in the rhizosphere, and the increase in proline content of plants (Table 3) may explain the improvement in the plant water status, positively reflected in the RWC.

Plants exposed to NaCl had increased Na content and reduced Ca content and Ca/Na ratio (Table 4). Na content in pea plants subjected to NaCl showed an increase of 7138% as compared to the unstressed control plants. On the other hand, Ca content and Ca/Na ratio in NaCl-stressed plants revealed a decrease of 12 and 99%, respectively, as compared to the unstressed control plants. Table 4 also shows that plants treated with calcium paste exhibited a highly significant decrease in Na content, and showed a significant increase in Ca content and Ca/Na ratio. These results may be explained on the basis of calcium paste components among the calcium ions, and act as antagonizers, which displace Na ions. However, the seed pretreatment with calcium paste resulted not only in the reduced inhibitory effects of NaCl, but also the further enhanced Ca/Na ratio and the reduced Na content.

The results of the assay of carbonic anhydrase (CA) and nitrate reductase (NR) revealed a drastic reduction in the activity of these enzymes in NaCl-treated plants when

Table 4. Effect of seed application with calcium paste on the leaf calcium (Ca) and sodium (Na) contents (mg g^{-1} dry weight) and Ca/Na ratio as well as on carbonic anhydrase (CA; units mg protein^{-1}) and nitrate reductase (NR; $\mu\text{M NO}_2 \text{ h}^{-1} \text{ g fresh weight}^{-1}$) activities in pea plants grown under salinity stress ($n = 10$).

Treatment	Parameter				
	Ca	Na	Ca/Na ratio	CA	NR
Control	12.8 ^e	0.34 ^d	37.6 ^c	2.5 ^c	4.1 ^c
CW*	17.2 ^c	0.18 ^d	95.6 ^b	2.8 ^b	4.6 ^b
CWH*	22.6 ^a	0.12 ^d	188.3 ^a	3.2 ^a	5.1 ^a
NaCl (150 mM)	11.3 ^e	24.61 ^a	0.5 ^e	1.4 ^d	2.0 ^e
NaCl + CW	15.4 ^d	10.84 ^b	1.4 ^e	2.3 ^c	3.7 ^d
NaCl + CWH	20.2 ^b	3.92 ^c	5.2 ^d	3.1 ^a	4.8 ^{ab}

*CW, Calcium paste consists of CaSO_4 + wheat bran at the ratio 1:5 (w/w). *CWH, Calcium paste consists of CaSO_4 + wheat bran + humic acid at the ratio 2:10:1 (w/w/w).

Table 5. Effect of seed application with calcium paste on the green yield components of pea plants grown under salinity stress ($n = 20$).

Treatment	Parameter		
	Green pod yield pot^{-1} (g)	100-seed weight (g)	Green seed yield pot^{-1} (g)
Control	85.9 ^b	18.4 ^b	40.1 ^c
CW*	92.3 ^a	20.6 ^a	45.3 ^b
CWH*	98.4 ^a	21.4 ^a	49.9 ^a
NaCl (150 mM)	14.8 ^e	10.7 ^d	5.6 ^f
NaCl + CW	39.9 ^d	13.8 ^c	14.0 ^e
NaCl + CWH	46.8 ^c	17.7 ^b	19.9 ^d

*CW, Calcium paste consists of CaSO_4 + wheat bran at the ratio 1:5 (w/w). *CWH, Calcium paste consists of CaSO_4 + wheat bran + humic acid at the ratio 2:10:1 (w/w/w).

compared with the control plants (Table 4). The activity of these enzymes (CA and NR) was not only restored by calcium paste in NaCl-treated plants, but also increased by 24 and 17%, respectively over the levels observed in unstressed control plants. Plants treated with calcium paste, particularly CWH also showed a significant increase in CA and NR activities. CA catalyses the reversible interconversion of HCO_3^- and CO_2 in the leaves and regulates the availability of CO_2 to Rubisco (Badger and Price, 1994). The activity of NR plays a pivotal role in the supply of nitrogen, and the growth and productivity of plants (Anuradha and Rao, 2009). The activity of NR is known to depend on the concentration of its substrate (NO_3^-) (Hayat et al., 2009). The decrease in NR activity in saline-stressed plants serves as a biochemical adaptation to conserve energy by stopping nitrate assimilation at the initial stage. However, application of calcium paste was found to be beneficial in enhancing the activities of NR and CA.

Results in Table 5 show that, plants treated with NaCl revealed a reduction in green pod yield pot^{-1} (3 plants pot^{-1}), 100-seed weight and green seed yield pot^{-1} by 83, 42 and 86%, respectively, as compared to the unstressed control plants. However, treatment of pea plants under stress

with calcium paste assuaged the inhibitory effect of salt stress on plants and significantly increased the yield components. CWH was found to be highly effective in increasing the green pod yield pot^{-1} , 100-seed weight and green seed yield pot^{-1} by 216, 65 and 255%, respectively, as compared to stressed control. In the present study, exogenous calcium (a calcium paste component) reversed the detrimental effect of NaCl stress on photosynthesis, growth and antioxidant system in pea plants, thus, positively reflected in the increase in the yield components. The positive influences of humic acids on plant growth and consequently yield components, which seem to be concentration-related, could be mainly due to hormone-like activities of the humic acids through their involvement in cell respiration, photosynthesis, oxidative phosphorylation, protein synthesis, antioxidant and various enzymatic reactions (Zhang and Schmidt, 2000; Zhang et al., 2003).

Conclusion

The results of this study clearly indicated that, the application of calcium paste, particularly CWH [CaSO_4 + wheat bran + humic acid at a ratio of 2:10:1 (w/w/w),

respectively] seemed to be useful to overcome the inhibition caused by NaCl stress. Seed pretreatment with CWH resulted in the improvement of growth, yield, and leaf pigment content, water and nutrient status and the activities of CA and NR in pea plants grown under NaCl stress. These results suggest that CWH application regulates the response of plants to the NaCl stress and could be used as a growth regulator during vegetative growth period to improve plant growth under salinity stress conditions.

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