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# Impact of Ascorbic Acid on Seed Germination, Seedling Growth, and Enzyme Activity of Salt-Stressed Fenugreek

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## ABSTRACT

Fenugreek (*Trigonella foenum-graecum*) seeds soaked in ascorbic acid had increased germination, seedling shoot length and total chlorophyll under salt stress as compared with seeds not treated with ascorbic acid. Root length, fresh weight, dry weight, proline, and catalase activity (CAT) increased in salt stressed seedling in which seeds were not treated with ascorbic acid. In seeds treated with ascorbic acid, the salt stress effect on CAT activity was decreased. Ascorbic acid pretreatment of seeds counteracted the decrease in ascorbate oxidase (AO) induced by salt stress, but appeared to act synergistically with salt stress to decrease proline dehydrogenase. The application of ascorbic acid to fenugreek seed apparently increased antioxidant activity, leading to an increase in resistance to salt stress. Salt stress and the ascorbic acid treatment of seeds led to metabolic changes in seedlings as evidenced by changes in peroxidase (Prx) and esterase (Est) isozymes associated with increases in salt and ascorbic acid concentrations. Such changes could account for increases in seedling vigor with ascorbic acid seed treatment and the ability of the seedling to grow in the presence of a salt stress.

## INTRODUCTION

Fenugreek (*Trigonella foenum-graecum*) is grown as a medicinal and food plant in the Mediterranean region and several other Middle Eastern countries. Most of the land areas in which fenugreek is cultivated, however, are subject to desertification and have relatively high salt levels as a consequence of low and random precipitation and incorrect irrigation practices. In addition, many other areas of the world contain arid and semi-arid soils and water resources that are too saline for growth of common economic crops.

Salt stress, similar to many abiotic stress factors, is known to induce oxidative damage to plant cells from reactive oxygen species that affect the physiology and biochemistry of plants and that can lead to a reduction in plant yield (Azevedo-Neto et al., 2006). The reactive oxygen species can damage membranes and other essential macromolecules, such as photosynthetic pigments, proteins, DNA, and lipids (Fahmy et al., 1998). For stress protection, plants have developed enzymatic and non-enzymatic scavenging mechanisms for the reactive oxygen species (Demiral and Turkan, 2005). These scavenging mechanisms, such as the production of catalase to reduce hydrogen peroxide (Hernandez et al., 2000), enable the plant to maintain growth under stress conditions.

Ascorbic acid, an abundant, relatively small molecule in plants, plays multiple roles in plant growth, functioning in cell division, cell wall expansion, and other developmental processes (Asada, 1999; Conklin, 2001; Pignocchi and Foyer, 2003). In addition, ascorbic acid is a key substance in the network of plant antioxidants, including glutathione and enzymatic antioxidants that detoxify $H_2O_2$ to counteract oxygen radicals produced by the Mehler reaction and photorespiration (Noctor and Foyer, 1998). Yet, little information is available on the effect of ascorbic acid on salt stress or enzyme activity in fenugreek plants.

The objective of the present investigation was to determine the effects of ascorbic acid and salt stress on seed germination, seedling development, and metabolic activity in fenugreek.

## MATERIALS AND METHODS

**Plant material.** Fenugreek, *Trigonella foenum graecum*, cv. Giza-3, was used in this study. The seeds, obtained from the Legume Research Section of the Agriculture Research Center, Giza, Egypt, were surface sterilized by immersion in 0.5% sodium hypochlorite (NaOCl) solution for 5 min to prevent fungal infections and then washed three times with sterile, distilled water to remove any NaOCl residue. After washing, the seeds (1 g $L^{-1}$) were pretreated with ascorbic acid by soaking for 24 h at room temperature in an ascorbic acid solution at 0, 50, or 100 $mgL^{-1}$.

**Experimental.** To determine the growth and metabolic effects of ascorbic acid seed treatment on fenugreek seeds and seedlings under a salt stress, the seeds pretreated with ascorbic acid were exposed to 0, 50, and 100 mM NaCl during germination and seedling development.

Upon removal from the ascorbic acid pretreatment, 50 randomly selected seeds from each ascorbic acid treatment were immediately transferred into a sterile Petri dish (150 mm in diameter x 15 mm deep) containing two sheets of sterile Whatman No. 1 filter paper that had been premoistened with 10 mL of a NaCl solution. The Petri dishes, containing the seeds scattered on the upper surface of the moistened filter paper, were moved to a controlled environment chamber at 20 ± 2 $^oC$ for germination under a 18 h light-6 h dark cycle. The light cycle was from a mixture of incandescent bulbs and fluorescent tubes with a PAR = 135 μmol $m^{-2}s^{-1}$ and R-FR ratio = 1.92. All solutions were made with distilled water.

Seed germination was observed daily with fresh salt solution added to the Petri dishes as necessary to maintain moisture levels. Measurements of seedling vigor and metabolic activity were made at 14 days after transfer of the seeds to the Petri dishes. Seedling vigor was determined using the percent seed germination (ISTA, 1999), seedling stem and root length, seedling fresh and dry weight of ten randomly selected seedlings, and the seedling vigor index (seedling length in cm x germination percentage (ISTA, 1999). Dry weights were determined after drying the plant tissue to a constant weight in a hot air oven at 85$^o$C for 12 h (Krishnasamy and Seshu, 1990).

Metabolic activity within the seedlings was determined by measuring the chlorophyll and proline content and catalase, ascorbate oxidase, and proline dehydrogenase activity. The chlorophyll content of the seedlings was measured using the spectrophotometric method described by Hipkins and Baker (1986). Briefly, 3 mL of 100% methanol were added to approximately 50 mg of the plant leaf tissue contained in 5 mL vials covered with aluminum foil. The darkened vials containing the tissue were stored at 23$^o$C for 2 h, the contents were mixed, the methanol fraction was decanted, and the absorbance of the decanted methanol was measured at 650 and 665 nm in a Spectronic Gensys spectrophotomer (Thermo Scientific, Barrington, IL). Total chlorophyll was calculated using the formula: Chlorophyll (μg/mL) = $25.8 \times A_{650} + 4.0 \times A_{665}$ and then converted to mg chlorophyll/g plant tissue.

Free proline content of the plant tissue was determined according to the method described by Bates et al. (1973). Approximately 0.5 g of leaf tissue was homogenized in 3% sulphosalycylic acid, filtered to remove debris from the filtrate, and then mixed with ninhydrin reagent and acetic acid before heating in a water bath at 100$^o$C for 1 h. The reddish color complex that formed was mixed with toluene for reading the absorbance in the previously described spectrophotomer at 520 nm for comparison of the absorbance with that of a standard curve of proline concentrations.

Enzymes were extracted from 0.5 g leaf samples homogenized in a pre-chilled pestle and mortar containing ice cold 0.1 M phosphate buffer (pH 7.5) and 0.5 mM EDTA. Each homogenate was transferred to centrifuge tubes and centrifuged at 4°C in a Sorval model T21 (Thermo Scientific, Waltham, MA) refrigerated centrifuge for 15 min at 15,000 x g. The supernatant was decanted and used for measuring enzyme activity assays (Esfandiari et al., 2007) and total protein. Catalase activity was determined according to the method used by Aebi (1984) in which the disappearance $H_2O_2$ in a reaction mixture containing 0.3 mL 3% $H_2O_2$, 2.5 mL of 0.05 M phosphate buffer (pH 7), and 2.5 mL of plant extract is monitored by the decrease in absorbance at 240 nm.

Ascorbate oxidase activity was assayed at 25°C by following the decrease in absorbance of the reaction mixture at 265 nm using previously described spectrophotometer. The reaction mixture consisted of 0.05 M potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.002% metaphosphoric acid, 0.15 mM L-ascorbic acid, and enzyme solution in a final volume of 3.0 mL according to the method of Oberbacher and Vines (1963). Enzyme activity was defined as the amount of enzyme that oxidized 1 mol of ascorbic acid/min.

Proline dehydrogenase was assayed by following NADP+ reduction at 340 nm in a 0.15 M $Na_2CO_3$–HCl buffer (pH 10.3) containing 15 mM L-proline and 1.5 mM NADP+ (Ruiz et al., 2002).

Total protein content of leaf samples was determined using the Bradford assay (Bradford, 1976). Bradford dye regent (Bio-Rad, Hercules, CA) was prepared by diluting the dye concentrate with distilled water in a 1:4 ratio. A total of 5 mL of the diluted dye was added to test tubes containing 100 µL samples of the protein extract and buffer. The tubes containing the extract and dye were subsequently incubated at room temperature for 5 min, thoroughly mixed, and the color change determined spectrophotometrically at 595 nm for comparison with the color change in standardized BSA samples (Sigma-Aldrich, Inc., St. Louis, MO).

Isozymes of esterase and peroxidase associated with the ascorbic acid seed treatment and with the salt stress were separated via 8% native polyacrylamide gel electrophoresis (Native-PAGE) using the procedures outline by Wendel and Weeden (1989). The isozymes were extracted from five seedling leaves using an extraction buffer (1:3, w:v, tissue:buffer) consisting of 0.61 g of 50 mM Tris-HCl buffer, pH 7.5 (containing 5 mL of 5% glycerol, 100 µL of 14 mM mercaptoethanol made to 100 mL. Each sample was vortexed for 15 sec and centrifuged at 14,860 g for 10 min in a refrigerated centrifuge (Model 5280, Eppendorf, Inc., Hauppauge, NY). The supernatant was stored at -18 °C until the isozymes were separated on the gel and then stained overnight in the dark at 37 °C, following the procedure of Jonathan and Wendel (1990) (Table 1).

The stained isozyme gels were washed with two to three times with water and then placed in a fixative solution 9 parts ethanol and 11 parts glacial acetic acid for 24 h. After removing from the fixative solution, the gels were rinsed twice with water and then scanned using a Gel Doc-2001 gel documentation system (Bio-Rad Laboratories, Inc., Hercules, CA). The densitometric scanning of the bands was done in three directions (length, width, and intensity) to ensure full recognition of each band. Accordingly, relative amounts were quantified and scored.

Table 1. The isozyme staining solutions.

| Isozyme | Staining solution |
|---|---|
| Peroxidase | 1 M sodium acetate, pH 4.7, methanol,<br>3,3,5,5- tetramethyl benzidine (TBMZ)<br>0.30 % $H_2O_2$ (Graham et al., 1964) |
| Esterase | 100 mM $Na_3PO_4$, α-naphthyl acetate,<br>Fast blue RR salt (Fisher Scientific, Inc.,<br>Jonthan and Wendell, 1990) |

**Statistical analysis.** The experimental trials were arranged in a completely randomized design with three replicates. All measurements were subjected to a statistical analysis using an analysis of variance (ANOVA) for a completely randomized design as described by Gomez and Gomez (1984).

## RESULTS

While a salinity stress significantly reduced germination of fenugreek seeds at both the 50 and 100 mM NaCl level, pretreatment of seeds with ascorbic acid before exposure to salt stress, countered much of the salinity stress that reduced seed germination (Table 2). Seed germination increased from 66% in a 100 mM NaCl solution to 82% at the same level of salt stress in seeds pretreated with 100 mgL$^{-1}$ of ascorbic acid. Pretreatment of seeds with ascorbic acid only (no salt stress) had essentially no effect on germination.

In addition to the reduction in seed germination, fenugreek seedling shoot length and seedling vigor in were significantly reduced by salt stress. The salt stress, however, resulted in increases in root length and fresh and dry weights of seedlings from the seeds that germinated under salt stress. An ascorbic acid only treatment of seeds resulted in increased root length, seedling length, and fresh weight similar to that of a salt stress, but dry weight was not increased and seedling vigor was increased.

Treatment of salt-stressed fenugreek seeds with ascorbic acid altered the development of seedlings. The increases in root length, seedling length, and fresh weight stimulated by exposure to salt stress were reduced to growth levels observed in plants not exposed to salinity or ascorbic acid.

Both chlorophyll and proline content were affected by the application of salt stress and ascorbic acid (Table 3). In general, salt stress reduced the level of chlorophyll in leaves, while ascorbic acid increased the level of chlorophyll in the leaf tissue. Seeds treated with ascorbic acid and subjected to salt stress were able to produce seedlings with a higher level of chlorophyll in the leaves than seedlings not exposed to ascorbic acid. Treatment of fenugreek seeds with ascorbic acid caused a slight increase in proline content of leaf tissue, but salt stress essentially quadrupled proline levels.

Catalase activity in the leaves of seedling exposed to salt stress was significantly increased and ascorbic oxidase and proline dehydrogenase were decreased as compared with seedlings not exposed to salt stress. The pretreatment of seeds with ascorbic acid (no salt stress) had no apparent effect on any of the tested enzyme activity in seedling leaves. The ascorbic acid seed treatment, however, reduced the effect of salinity on catalase activity in the leaves and limited the effect of salinity on the activity of ascorbic oxidase. The ascorbic acid pretreatment enhanced the decrease in proline dehydrogenase activity induced by salt stress.

Differences in the number and pattern of esterase and peroxidase isozymes appeared in salt stressed seedlings as compared with control seedlings (no salt, no ascorbic acid pretreatment) (Tables 4 and 5). While three esterase isozyme (Rf 278, 401, and 662) appeared in the control seedlings (no salt and no ascorbic acid pretreatment), band Rf 401 was not present in seedlings treated with salt. The esterase isozyme band at Rf 0.278 was missing in seedlings that developed from seeds pretreated with ascorbic acid at the 100 mgL$^{-1}$ level. An additional esterase isozyme bands appeared in seedling treated with 100 mgL$^{-1}$ ascorbic acid at 50 (Rf 235) and 100 mM NaCl (Rf 0.147).

Similarly, peroxidase isozyme bands at Rf 212, 299, and 407, were present in the control seedling, but not in the salt-stressed plant tissue. The peroxidase isozymes in the control samples were missing in the salt stressed samples. New salt induced bands appeared at Rf 0.149, 0.198, 0.203, 0.256, and 0.407.

Table 2. The effect of ascorbic acid on salt-stressed seed germination and seedling development in fenugreek.

| Ascorbic acid[1] (mg L$^{-1}$) | Salinity (mM NaCl) | Germination (%) | Shoot length (cm) | Root length (cm) | Seedling length (cm) | Fresh weight (g) | Dry weight (g) | Seedling vigor index |
|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 93 | 3.7 | 4.3 | 8.0 | 0.09 | 0.015 | 751 |
| | 50 | 80 | 3.1 | 4.8 | 7.9 | 0.13 | 0.020 | 613 |
| | 100 | 66 | 2.7 | 6.6 | 9.3 | 0.18 | 0.031 | 628 |
| 50 | 0 | 91 | 3.6 | 5.2 | 8.8 | 0.13 | 0.016 | 814 |
| | 50 | 85 | 3.4 | 4.2 | 7.6 | 0.12 | 0.018 | 653 |
| | 100 | 79 | 3.2 | 4.7 | 7.9 | 0.14 | 0.029 | 633 |
| 100 | 0 | 90 | 3.8 | 5.5 | 9.3 | 0.14 | 0.015 | 851 |
| | 50 | 87 | 3.6 | 4.1 | 7.7 | 0.12 | 0.017 | 670 |
| | 100 | 82 | 3.4 | 4.3 | 7.7 | 0.12 | 0.026 | 641 |
| L.S.D.5% | - | 2 | 0.1 | 0.1 | 0.4 | 0.04 | 0.003 | 35 |

[1]Seeds were pretreated with indicated level of ascorbic acid and then subjected to the indicated salt stress for 14 days.

Table 3. The effect of ascorbic acid on chlorophyll, proline, and enzyme activity in fenugreek seedlings exposed to a salt stress.

| Ascorbic acid[1] (mg L$^{-1}$) | Salinity (mM NaCl ) | Chlorophyll (mg/g F.Wt.) | Proline (mmoles/g F.Wt. | Catalase (U/mg protein) | Ascorbate oxidase (U/mg protein) | Proline dehydrogenase (U/mg protein) |
|---|---|---|---|---|---|---|
| 0 | 0 | 0.758 | 6.2 | 15.5 | 126.8 | 38.1 |
| | 50 | 0.422 | 18.4 | 33.6 | 88.0 | 36.2 |
| | 100 | 0.383 | 23.5 | 50.6 | 75.9 | 35.8 |
| 50 | 0 | 0.798 | 7.2 | 16.1 | 131.9 | 22.7 |
| | 50 | 0.644 | 26.2 | 25.3 | 121.2 | 17.2 |
| | 100 | 0.564 | 35.1 | 35.2 | 111.5 | 12.6 |
| 100 | 0 | 0.851 | 7.1 | 16.5 | 134.1 | 19.2 |
| | 50 | 0.690 | 35.1 | 20.8 | 128.1 | 13.2 |
| | 100 | 0.536 | 38.4 | 25.1 | 118.4 | 10.1 |
| L.S.D.5% | - | 0.002 | 0.4 | 0.8 | 1.7 | 0.7 |

[1]Seeds were pretreated with indicated level of ascorbic acid and then subjected to the indicated salt stress for 14 days.

Table 4. Esterase isozymes.

| Visible bands[1] | Salt level (mM NaCl) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | |
| | Ascorbic acid (mg $L^{-1}$) | | | Ascorbic acid (mg $L^{-1}$) | | | Ascorbic acid (mg $L^{-1}$) | | |
| Rf | 0 | 50 | 100 | 0 | 50 | 100 | 0 | 50 | 100 |
| 0.147 | - | - | - | - | - | - | - | - | + |
| 0.235 | - | - | - | - | - | + | - | - | - |
| 0.278 | + | + | - | + | + | + | + | + | + |
| 0.401 | + | + | + | - | - | - | - | - | - |
| 0.662 | + | + | + | + | + | + | + | + | + |

[1]Rf = retention factor; band present = +; band absent = -.

Table 5. Peroxide isozymes

| Visible bands[1] | Salt level (mM NaCl) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | |
| | Ascorbic acid (mg $L^{-1}$) | | | Ascorbic acid (mg $L^{-1}$) | | | Ascorbic acid (mg $L^{-1}$) | | |
| Rf | 0 | 50 | 100 | 0 | 50 | 100 | 0 | 50 | 100 |
| 0.141 | - | + | + | - | - | - | - | - | - |
| 0.149 | - | - | - | - | - | - | - | - | + |
| 0.198 | - | - | - | - | - | - | - | - | + |
| 0.203 | - | - | - | - | - | + | - | + | - |
| 0.212 | + | + | + | - | - | - | - | - | - |
| 0.256 | - | - | - | + | + | + | + | + | + |
| 0.299 | + | + | + | - | - | - | - | - | - |
| 0.397 | + | + | - | - | - | - | - | - | - |
| 0.407 | - | - | + | - | + | + | + | + | + |

[1]Rf = retention factor; band present = +; band absent = -.

## DISCUSSION

Soil **s**alinity is an environmental stress that can reduce seed germination, plant growth, and plant yield and plant constituency (Baâtour et al., 2011;Seeman and Critchley, 1985). While these plant developmental reductions observed in salt stressed plants may result from the low water potential or nutritional imbalances, a salt stress could also interfere with metabolic processes, causing an increase in reactive oxygen species that can inhibit growth and development. Smirnoff (2005) noted that germination, fresh and dry weights, and chlorophyll increased when ascorbic acid was added as antioxidant to the media.

Germination of fenugreek seeds under salt stress were significantly reduced in direct relation to the level of salt stress, an effect that could possibly be due to reduced water absorption by seeds or reduced seed metabolism due the presence of salt. The germination of fenugreek seeds pretreated with ascorbic acid before application of the salt stress was, however, very similar to seeds not exposed to a salt stress, suggesting the effect of the salt was primarily due to metabolic interference as opposed to a reduction in water absorption.

The decrease in shoot length and increase in root length suggests stimulation of root growth by salt stress respiration. The stimulation of respiration in seedling roots increases growth (length and dry weight) as compared with growth of the shoots and has been recognized in other plants (Moud and Maghsoudi, 2008; Weimberg, 1970). Pretreatment of seeds with ascorbic acid produced the highest levels of seedling vigor and appeared to counteract any growth increase at the 50 mM salt concentration, but not at the 100 mM salt concentration.

The observed increase proline accumulation in fenugreek seedlings under salt stress could be expected as increased synthesis of proline is a common metabolic reaction to plants under stress. Similar increases in proline have been observed in other plants subjected to a salt stress (Misra and Gupta, 2005), acting as an osmotic regulator and antioxidant substrate. (Kishor et al., 1995). The lower levels of chlorophyll observed in the fenugreek seedlings under salt stress is consistent with that observed in other plants under salt stress, such as wild potato clones (Mohamed et al., 2010) and wheat seedlings (Ruan et al., 2002), and most probably results from oxidative damage induced by the salt stress (Jiang et al., 1994).

Although the formation of reactive oxygen species (ROS) occurs naturally as a by-product of metabolism, environmental stresses are known to increase ROS to toxic levels (Mittler et al., 2004), overwhelming protective ROS scavenging mechanisms and resulting in severe damage to cellular structures and cell death (Sharma et al., 2012). The observed increases in catalase activity under salt stress and the observed decrease in catalase activity in salt-stressed seedling pre-treated with the antioxidant ascorbic acid strongly supports the presence of enhanced ROS levels in tissue under salt stress. The ascorbic acid neutralizes the ROS, protecting the plant tissue from harmful effects of ROS and there-by improves plant resistance to salt stress.

Changes in the number and/or activity of esterase and peroxidase isozymes in salt stressed seedlings from those in control seedlings not under salt stress suggest the plant is making adjustments in metabolic reactions in an attempt to mitigate the salt stress. The changes in the esterase and peroxidase isozymes in seedlings growing from seeds pretreated with ascorbic acid may be responsible in part for the increased tolerance of fenugreek seedlings to salt stress (Hassanein, 1999). The present findings suggest that salt stress may be regulated by physiological processes, and that manipulating these processes could enhance plant salt stress. Elucidating the mechanisms controlling the levels of peroxidase and esterase isozymes induced in response to salt stress and ascorbic acid may provide insights into metabolic regulatory systems susceptible to salt beyond those associated with antioxidant affects.

Earlier investigations have shown that salt tolerant plant cultivars have a more active antioxidant system than salt sensitive cultivars of the same species (Raza et al., 2006; Ashraf and Foolad, 2007). From these observations, exogenous application of antioxidants has gained considerable attention as an possible approach to ameliorate the adverse effects of salinity stress on plants (Gaddallah, 2000; Khan et al., 2003).

This study clearly demonstrated that salt stress on seedlings could by significantly reduced by the use of an ascorbic acid pretreatment of seeds. The reduction in harmful effects of salt on seedling growth should prove useful to growers. The seed pretreatment with ascorbic acid could be applied by growers just before seeding and enable the plants to establish in saline soils.

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