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Effect of Arbuscular Mycorrhizal Inoculation on Salt-induced Nodule Senescence in *Cajanus cajan* (Pigeonpea)

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Abstract Many physiological and biochemical plant processes affected by salt stress trigger premature nodule senescence and decrease their ability to fix nitrogen. The objective of this study was to evaluate the role of arbuscular mycorrhiza (AM) in moderating salt-induced premature nodule senescence in *Cajanus cajan* (L.) Millsp. Greenhouse experiments were conducted in which the plants were exposed to salinity stress of 4, 6, and 8 dSm^{-1}. Various parameters linked to nodule senescence were assessed at 80 days after sowing. Nodulation, leghemoglobin content, and nitrogenase enzyme activity measured as acetylene-reducing activity (ARA) were evaluated. Two groups of antioxidant enzymes were studied: (1) enzymes involved in the detoxification of O_2^- radicals and H_2O_2, namely, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), and (2) enzymes that are important components of the ascorbate glutathione pathway responsible for the removal of H_2O_2, namely, glutathione reductase (GR) and ascorbate peroxidase (APOX). Exposure of plants to salinity stress enhanced nodule formation; however, nodule growth suffered remarkably and a marked decline in nodule biomass, relative permeability, and lipid peroxidation was observed. Leghemoglobin content and ARA were reduced under saline conditions. AM significantly improved nodulation, leghemoglobin content, and nitrogenase activity under salt stress. Activities of SOD, CAT, APOX, POX, and GR increased markedly in mycorrhizal-stressed plants. A synthesis of the evidence obtained in this study suggests a correlation between enhanced levels of antioxidant enzyme activities, reduced membrane permeability, reduced lipid peroxidation, and improved nitrogen-fixing efficiency of AM plants under stressed and unstressed conditions. These factors could be responsible for the protective effects of mycorrhiza against stress-induced premature nodule senescence.

Keywords Antioxidants · Arbuscular mycorrhiza · Leghemoglobin · Membrane stability · Nitrogenase · Nodule · Salinity · Senescence

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Introduction

Salinity is an ever-present threat to crop yield. The responses to salt stress comprise an array of changes at the molecular, biochemical, and physiological levels. Legume root nodules are symbiotic organs wherein rhizobial bacteria fix atmospheric nitrogen to supply the plant with ammonium. In the plant, ammonium is incorporated into essential macromolecules such as amino acids and proteins that drive plant growth, development, and crop yields. Root nodules not only make a crucial contribution to the N economy of leguminous crops, but they also enhance the N content of the soil and thus have a key role in environmentally friendly agricultural practices (Puppo and others 2005). Because of their ability to establish endosymbiosis with rhizobia, leguminous plants can grow under nitrogen-limiting conditions. This attribute makes legumes interesting candidates for improving saline soil fertility and helping to reintroduce agriculture to these lands. However, legumes are very sensitive to saline stress. The accumulation of nitrogen in seeds depends largely on efficient nitrogen fixation in the root nodules, which is a sensitive target for salt stress.

The senescence process in nodules is characterized by membrane damage, particularly to the symbiosome membrane (Puppo and others 1991; Hernandez-Jimenez and others 2002). Many studies have implicated reactive oxygen species (ROS) and antioxidants in nodule senescence (Escuredo and others 1996; Evans and others 1999; Becana and others 2000; Matamoros and others 2003). Salt stress can trigger premature nodule senescence (Gogorcena and others 1997; Gonzalez and others 1998; Matamoros and others 1999). In addition to the physiological responses, salinity induces acceleration of lytic activities, formation of green pigments from leghemoglobin (Lb) (Sarath and others 1986), and the loss of N_2 fixation (Delgado and others 1994), causing premature root nodule senescence. This induced nodule senescence develops much more rapidly than developmental senescence and presents features of oxidative stress and plant cell death (Puppo and others 2005).

In functional nodules there are a number of processes that contribute to the high ROS levels. First, reactive oxygen species (Santos and others 2001; D'Haeze and others 2003; Matamoros and others 2003; Shaw and Long 2003) are part of the repertoire of signals that contribute to the establishment of the legume-rhizobia symbiosis. Second, nodules have high rates of respiration due to the extensive energy demands of N_2 fixation, which results in a high flux of O_2 into the nodule and, hence, inevitably leads to ROS formation as many electron transfer components, including ferredoxin, uricase, and hydrogenase, are susceptible to auto-oxidation resulting in superoxide formation (Dalton 1995). The auto-oxidation of oxygenated Lb to ferric Lb yields O_2^-. This radical can dismutate to H_2O_2, which in turn may attack Lb, releasing catalytic Fe and producing the highly toxic $HO^\bullet$ radical (Puppo and Halliwell 1988; Hernandez-Jimenez and others 2002). Legume nodules possess a number of antioxidant enzymatic mechanisms to avert or limit the toxicity of ROS (Kunert and Foyer 1994; Comba and others 1998). Antioxidant enzymes either catalyze such reactions or are involved in direct processing of ROS like superoxide dismutases (SODs; present in bacteroids and nodule cytosol, mitochondria; catalyze the dismutation of O_2^- to O_2 and H_2O_2), catalases (CATs; present in bacteroids and peroxisomes of nodules; dismutate two molecules of H_2O_2 into H_2O and O_2), peroxidases (POXs; present in host cell; catalyze the reduction of H_2O_2 to H_2O), ascorbate peroxidase (APOX; present in nodule cytosol; catalyze the reduction of H_2O_2 to water using the reducing power of ascorbate), glutathione reductase (GR; present in nodule cytosol; catalyzes the reduction of oxidized glutathione to reduced glutathione using NADPH as the reducing cofactor) (Foyer and Halliwell 1976; Noctor and Foyer 1998; Becana and others 2000; Tavares and others 2007). Premature stress-induced senescence has been linked to the enhanced production of oxidants (leading to oxidative damage to biomolecules such as lipids and proteins) and the lowering of antioxidant defenses (Escuredo and others 1996; Gogorcena and others 1997).

In addition to intrinsic protective systems of plants against stress, plants grow in association with arbuscular mycorrhizal fungi that are known to exist in saline environments, where they improve tolerance of plants to salinity (Juniper and Abbott 1993; Al-Karaki 2000; Al-Karaki and others 2001). Arbuscular mycorrhizal (AM) symbiosis can alleviate drought-induced nodule senescence in legume plants (Ruiz-Lozano and others 2001; Porcel and others 2003). However, reports on mycorrhizal amelioration of salt stress-induced premature nodule senescence are lacking.

Pigeonpea (*Cajanus Cajan* [L.] Millspaugh) is one of the major grain legume (pulse) crops of the tropics and subtropics. Pigeonpea belongs to the subtribe Cajaninae of the agriculturally most important tribe Phaseoleae under subfamily Papilionoideae of the family Fabaceae. In addition to its utilization as an annual crop, it can fit into agroforestry and shifting cultivation systems as a source of seed and forage for livestock and as a soil ameliorator. It can provide considerable residual benefit for the succeeding crops such as wheat (Johansen and others 1990). The crop has been traditionally grown as an intercrop or mixed crop with a number of cereals such as sorghum (*Sorghum bicolor* L.), pearl millet (*Pennisetum glaucum* L.), and maize (*Zea mays* L.). *Cajanus cajan* (pigeonpea) is generally observed to provide better yields than other crops in low-P soils, even without P fertilizer application, because of an extensive rooting habit and strong mycorrhizal development. Pigeonpea ranks sixth in area and production in comparison to other grain legumes such as beans, peas, and chickpeas. Its high sensitivity to salinity poses a major constraint to crop production in certain areas (Chauhan 1987). Worldwide, approximately 100 million ha of arable land are affected by salinity, which accounts for about 6–7% of the total arable area (Munns and James, 2003). In India, where 90% of the world's pigeonpea is produced, approximately 13.3 million ha of land are affected by salinity (Consortium for Unfavorable Rice Environment 2003).

The aim of the present work was to determine whether AM was effective in ameliorating the negative effects of salt stress in the nodules of pigeonpea. The investigations were thus directed to analyze various parameters related to premature nodule senescence (nitrogenase activity, leghemoglobin content, antioxidant enzyme activities, relative permeability of nodular membranes, lipid peroxidation) in unstressed and salt stressed nodules of AM inoculated and uninoculated plants.

Materials and Methods

AL201, an Extra Short Duration Pigeonpea (ESDP) cultivar, was used for study in the present investigations.

Plant Growth Conditions

The experiments were conducted from mid-June to October 2006 in a greenhouse of the Department of Botany, Panjab University, Chandigarh, India (30.5°N, 76.5°E and elevation 305–366 m). The minimum temperature ranged from 22°C to 29°C and maximum temperature ranged from 30°C to 37°C. The morning relative humidity was between 55% and 92% and afternoon relative humidity was between 42% and 81%.

Rhizobial and AM Inoculum

The seeds were rinsed with water and surface sterilized by dipping in 0.2% mercuric chloride ($HgCl_2$) solution for 2 min and repeatedly rinsed thereafter with sterilized water under aseptic conditions. Seeds were pretreated with a standard rhizobial inoculum of *Sinorhizobium fredii* AR-4 obtained from the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi, India. AM inoculum of *Glomus mosseae* was obtained from the Division of Microbiology, IARI. The AM spores were applied at 10 spores per seed (approximately 1500 spores/100 g media). Seeds were inoculated by placing the AM inoculum in the hole under the seed and covering with the soil.

The soil (mixture of sand and loam in ratio of 1:1) was obtained from the nearby agricultural fields. It was fumigated with methyl bromide under air-tight plastic sheets and the fumigant was allowed to dissipate for one week (Al-Raddad 1991). The pots were divided into equal lots of 25 pots (30 cm × 25 cm × 25 cm) for each treatment. Eight seeds were sown in each pot containing 7 kg of soil mixture, pH 7.2, electrical conductivity 1.0 dSm^{-1} and P content of 20 ppm. Seedlings were thinned to four after 6 days of emergence. When the seedlings were established (15 days after sowing), the plants were treated with saline solutions of three different concentrations, 4, 6, and 8 dSm^{-1} ECe. To obtain the desired saline concentration of the soil, a mixture of sodium chloride, calcium chloride, and sodium sulfate was prepared according to specifications of Richards (1954). The control plants (1 dSm^{-1}) were treated with distilled water only. Regular fortifications of saline solutions were made to maintain the desired soil salinity levels after monitoring the conductivity levels of the soils at weekly intervals with the help of an electrical conductivity (EC) meter until the end of the experiments. Parameters such as mycorrhizal infection, normalized nodule weight, leghemoglobin content, nitrogenase activity, relative permeability, lipid peroxidation, and antioxidant enzymes activities were studied at the preflowering stage of 80 days after sowing. Replicates for each experiment consisted of six pots selected from each of the treatments. Careful separation of the plants from the pots was done by gently washing out the soil with running tap water to avoid damage to the nodules. The plants, along with the adhering soil, were transferred to the sieve and roots and nodules were collected from the sieve and combined with the rest of the plant material. For dry weight measurements, the samples were dried in an oven at 70°C for 72 h.

Measurements

Mycorrhizal Infection

Mycorrhizal infection was estimated by the method of Phillips and Hayman (1970). The roots were cut and dipped in KOH solution for 24 h and then kept in HCl solution for 15–30 min. Staining solution containing cotton blue dye was added. The samples were kept for 24–36 h. The roots were cut into small pieces of approximately 2.5 cm and observed under a compound light microscope. Root pieces that contained even a single vesicle or arbuscules were considered infected. The percent root infection was calculated with the following formula:

$$\text{Percent root infection} = \frac{\text{Total number of infected roots}}{\text{Total number of roots observed}} \times 100$$

Leghemoglobin

The nodules were detached immediately after sampling and their leghemoglobin content was determined by the method of Hartree (1957), which is based upon the conversion of hematin to pyridine hemochromogen. A standard curve was prepared using graded concentrations of hemin.

Normalized nodule weight

Normalized nodule weight (NNW) was calculated by dividing the nodule dry weight (mg $plant^{-1}$) by the shoot dry weight (g $plant^{-1}$) (Olivera and others 2004).

Nitrogenase Activity

The acetylene–ethylene assay of nitrogenase activity was done by the method of Herdina and Silsbury (1990). The nitrogen-fixing complex (nitrogenase) of legumes is able to

reduce C_2H_2 to C_2H_4. The root system with intact nodules was incised from freshly separated plants and was incubated at room temperature in vials containing acetylene (C_2H_2) (10%, v/v) in air and sealed with serum caps. The samples were flushed with acetylene gas by gently shaking the bottles and were incubated for 1 h. The sample of 1 ml of gas from the incubation mixture was analyzed for ethylene in a PerkinElmer 8600 gas chromatograph equipped with a Porapak R column (Ligero and others 1986). From the standard values, the number of moles of ethylene produced in each case was calculated, the nodules were dried in an oven at 70°C for 24 h, and their dry weights were taken. The rate of enzyme activity was calculated as number of moles of ethylene produced per mg dry weight of nodules per hour (n mol ethylene mg dry wt.$^{-1}$ h^{-1}).

Relative Permeability

The relative permeability of the root plasma membrane was calculated as described by Zwiazek and Blake (1991). A sample of 2.5 g of fresh plant material was placed in 25 ml of deionized water. The electrolytic conductivity of the bathing solution was measured with a conductivity meter 10 min after soaking the plant material in water. The plant material was then heated to boiling. The bathing solution was cooled to room temperature and electrolytic conductivity measured again.

$$\text{Relative Permeability} = \frac{\text{Electrolyte conductivity of solution before heating}}{\text{Electrolytic conductivity of solution after heating}} \times 100$$

Superoxide Dismutase (SOD) Activity

The assay is based on formation of blue-colored formazone by nitroblue tetrazolium and the O_2^- radical, which absorbs at 560 nm, and the enzyme (SOD) decreases this absorbance due to reduction in the formation of the O_2^- radical by the enzyme (Dhindsa and others 1981). Plant samples frozen in liquid nitrogen were ground with 10 ml of extraction buffer. Brie was filtered and the filtrate was centrifuged and the supernatant was used as the enzyme source. Three milliliters of the reaction mixture contained 13.33 mM methionine, 75 µM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, 0.05 ml enzyme, and 0.95 ml distilled water. The reaction was started by adding 2 µM riboflavin and placing the tubes under two 15-W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as the control. Switching off the light and putting the tubes into the dark stopped the reaction. A nonirradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

Catalase (CAT) Activity

The catalase assay is based on the absorbance of H_2O_2 at 240 nm in the UV range. A decrease in the absorbance is recorded over a time period as described by Aebi (1984). The 3.0-ml reaction mixture consisted of 50 mM (pH 7.0) potassium phosphate buffer, 12.5 mM hydrogen peroxide, 50 µl enzyme, and distilled water to make a volume of 3.0 ml. Adding H_2O_2 started the reaction and a decrease in absorbance was recorded for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed. The initial and final contents of hydrogen peroxide were calculated by comparison with a standard curve drawn with known concentrations of hydrogen peroxide. Enzyme activity was calculated as the concentration of hydrogen peroxide reduced (initial reading – final reading = quantity of hydrogen peroxide reduced) per min per mg protein.

Peroxidase (POX) Activity

Peroxidase activity was assayed as the increase in optical density due to the oxidation of guaiacol to tetraguaiacol (Castillo and others 1984). The 3-ml reaction mixture contained 16 mM guaiacol, 2 mM H_2O_2, 50 mM phosphate buffer (pH 6.1), and 0.1 ml enzyme extract. Enzyme extract was prepared as in the case of SOD. The reaction mixture consisted of 50 mM (pH 6.1) phosphate buffer, 16 mM guaiacol, 2 mM H_2O_2, 0.1 ml enzyme, and 0.4 ml distilled water to make a final volume of 3.0 ml. Absorbance due to the formation of tetraguaiacol was recorded at 470 nm and enzyme activity was calculated as per the extinction coefficient of its oxidation product tetraguaiacol, $\epsilon = 26.6$ mM^{-1} cm^{-1}. Enzyme activity was expressed as µmol tetraguaiacol formed per min per g fr. wt. or per mg protein.

Ascorbate Peroxidase (APOX) Activity

The assay is based on the decrease in absorbance of ascorbic acid at 290 nm because of oxidation of ascorbic acid to monodehydroascorbic acid and dehydroascorbic acid (Nakano and Asada 1981). The enzyme extract was

prepared as in the case of SOD except the extraction buffer contained 1 mM ascorbic acid in addition to other ingredients. The 3-ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, 0.1 ml enzyme, and 0.7 ml distilled water to make a final volume of 3.0 ml. The reaction was started with the addition of 0.2 ml hydrogen peroxide. Decrease in absorbency for 30 s was measured at 290 nm in an UV-visible spectrophotometer. The initial and final contents of ascorbic acid were calculated by comparing with a standard curve drawn with known concentrations of ascorbic acid. Enzyme activity was calculated as concentration of ascorbic acid oxidized (initial reading – final reading = quantity of ascorbic acid oxidized) per min per mg protein.

Glutathione Reductase (GR) Activity

The assay of the enzyme is based on the formation of a red-colored complex by reduced glutathione with 5,5-dithiobis-2-nitrobenzoic acid (DTNB), which absorbs at 412 nm (Smith and others 1988). The enzyme extract was prepared as in the case of SOD. The reaction mixture contained 66.67 mM potassium phosphate buffer (pH 7.5) and 0.33 mM EDTA, 0.5 mM DTNB in 0.01 M potassium phosphate buffer (pH 7.5), 66.67 μM NADPH, 666.67 μM GSSG (oxidized glutathione), 0.1 ml enzyme extract, and distilled water to make a final volume of 3.0 ml. The reaction was started by adding 0.1 ml 20.0 mM GSSG. The increase in absorbance at 412 nm was recorded spectrophotometrically. The activity was expressed as total absorbance at 412 nm (ΔA_{412}) per mg protein per min.

Lipid Peroxidation

The level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) content (Heath and Packer 1968). Lipid peroxidation products such as malondialdehyde and fatty acid hydroperoxides, react with thiobarbituric acid and form a red-colored complex known as thiobarbituric acid reactive substance, which is taken as the measure of *in vivo* lipid peroxidation in plant tissue. This red-colored complex absorbs at 532 nm. A plant sample of 0.5 g was homogenized in 10 ml 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000*g* for 15 min. The supernatent was used for the estimation of TBARS contents. To a 1.0-ml aliquot of the supernatant 4.0 ml 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95°C for 30 min and then cooled in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbancy of the supernatant was recorded at 532 nm. Values of nonspecific absorption recorded at 600 nm were subtracted from the values recorded at 532 nm. The TBARS content was calculated according to its extinction coefficient $\epsilon = 155\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$.

All data were subjected to analysis of variance using one-way ANOVA and means were compared by Duncan's multiple-range test (Duncan 1955).

Results

Nodulation

Nodule number increased with saline treatments of 4 and 6 dSm^{-1} in AM-inoculated and uninoculated plants. However, a decline in nodule number was observed at 8 dSm^{-1} (Table 1). The inoculated plants showed significantly higher nodule numbers than uninoculated plants. Also, a color change from pink to brownish pink was observed indicating that although new nodules formed, they were not symbiotically active. The color change was observed much earlier in non-AM plants (8 weeks) than in AM-inoculated plants (10 weeks), indicating that nodule senescence had set in earlier in cases of stressed uninoculated plants than in stressed plants inoculated with *Glomus mosseae*. Although salt stress increased the number of nodules, a significant decline in nodule size and dry mass was observed under all the saline treatments compared to uninoculated controls (1 dSm^{-1}). The presence of fungal endophytes in the rooting medium significantly increased nodule dry mass under stressed and unstressed conditions. Normalized nodule weight (NNW) decreased with increase in saline concentrations, and the minimum NNW was observed under the highest saline concentration of 8 dSm^{-1}, suggesting more sensitivity of the nodules to salt than the aerial parts of the plants. *Glomus mosseae* significantly increased NNW under stressed and unstressed conditions when compared to uninoculated controls (Table 1).

Nitrogen Fixation

Exposing the plants to salt stress resulted in a sharp reduction in the leghemoglobin content in non-AM plants. However, significantly lesser damage to leghemoglobin protein was recorded in AM-inoculated stressed plants. Nodule activity in terms of acetylene-dependent ethylene production (ARA) (Table 1) followed the reduction in nodule dry mass and leghemoglobin content under salt stress. The efficiency of plant-*Rhizobium* symbiosis increased significantly and the symbiotic performance was better in AM-inoculated than the corresponding uninoculated plants under salt stress.

Table 1 Effect of AM Inoculation on Mycorrhizal Infection, Nodulation, Nitrogen Fixation, and Relative Permeability in Pigeonpea Under Salt Stress

Parameters measured	Control (C)	C+AM	4 dSm^{-1}	4 dSm^{-1} + AM	6 dSm^{-1}	6 dSm^{-1} + AM	8 dSm^{-1}	8 dSm^{-1} + AM
Mycorrhizal infection (%)	0.00	$93.22^{d} \pm 1.128$	0.00	$84.65^{c} \pm 0.926$	0.00	$82.15^{b} \pm 1.473$	0.00	$75.60^{a} \pm 1.527$
Dry weight of nodules (g $plant^{-1}$)	$0.232^{d} \pm 0.007$	$0.464^{f} \pm 0.008$	$0.151^{c} \pm 0.010$	$0.271^{e} \pm 0.006$	$0.074^{b} \pm 0.008$	$0.153^{c} \pm 0.006$	$0.035^{a} \pm 0.008$	$0.086^{b} \pm 0.011$
Number of nodules per plant	$25.21^{c} \pm 0.55$	$46.59^{g} \pm 0.49$	$29.37^{d} \pm 0.691$	$71.75^{h} \pm 0.651$	$31.55^{e} \pm 0.45$	$41.41^{f} \pm 0.57$	$18.33^{a} \pm 0.437$	$22.23^{b} \pm 0.49$
Leghemoglobin content (μg g^{-1} fr. wt.)	$127.77^{e} \pm 1.849$	$172.30^{g} \pm 1.963$	$97.98^{c} \pm 1.749$	$135.77^{f} \pm 1.998$	$87.58^{b} \pm 1.439$	$107.51^{d} \pm 2.159$	$29.50^{a} \pm 1.744$	$85.88^{b} \pm 1.358$
Normalized nodule weight (NNW) (mg NDW g^{-1} SDW)	$0.047^{e} \pm 0.0004$	$0.053^{f} \pm 0.0004$	$0.042^{d} \pm 0.0004$	$0.047^{e} \pm 0.0007$	$0.034^{b} \pm 0.0006$	$0.038^{c} \pm 0.0002$	$0.029^{a} \pm 0.0002$	$0.035^{b} \pm 0.0004$
Nitrogenase activity (n mol ethylene mg^{-1} nodule dry wt. h^{-1})	$0.103^{f} \pm 0.004$	$0.143^{g} \pm 0.001$	$0.077^{d} \pm 0.004$	$0.101^{f} \pm 0.005$	$0.068^{b} \pm 0.002$	$0.093^{e} \pm 0.002$	$0.019^{a} \pm 0.003$	$0.041^{b} \pm 0.002$
Relative permeability (%)	$27.38^{a} \pm 1.095$	$26.18^{a} \pm 0.922$	$46.01^{d} \pm 1.260$	$41.39^{b} \pm 1.139$	$51.27^{e} \pm 0.407$	$45.95^{c} \pm 1.517$	$58.42^{f} \pm 1.101$	$53.02^{e} \pm 1.670$

Each value is the mean of six replicates. Means followed by the same letter within a row are not significantly different at $p < 0.05$, as determined by Duncan's multiple-range test

Mycorrhizal Infection

The results pointed out that different levels of salt did not affect the colonizing ability of the fungi significantly (Table 1) because significant mycorrhizal infection was observed at different saline concentrations. Mycorrhizal colonization was more tolerant to salinity than plant-*Rhizobium* symbiosis as indicated by a greater decline in nodule biomass as well as nitrogenase activity under each treatment.

Relative Permeability

Salt stress increased nodule plasma membrane electrolyte permeability. Significantly higher damage to nodular cell membranes was recorded in stressed plants. The negative effects of salt stress, however were moderated in the presence of fungal endophyte.

Antioxidant Enzyme Activities

Increased levels of salt in the soils resulted in a general increment in the antioxidant enzyme activities of the nodules. SOD activity (Figure 1) was elevated in response to moderate (4 dSm^{-1}) and heavy (6 dSm^{-1}) salt stress. Saline dosages of 4 and 6 dSm^{-1} increased the SOD activity by 1.3 and 1.7 times in unionculated plants when compared to control plants at 1 dSm^{-1}. Significantly higher SOD activity (2.0 and 3.4 times) was observed in mycorrhizal plants at 4 and 6 dSm^{-1}, respectively, when compared to controls. The enzyme activity of AM-stressed plants was 1.4 and 2 times higher relative to non-AM-stressed plants at 4 and 6 dSm^{-1}, respectively. Increased

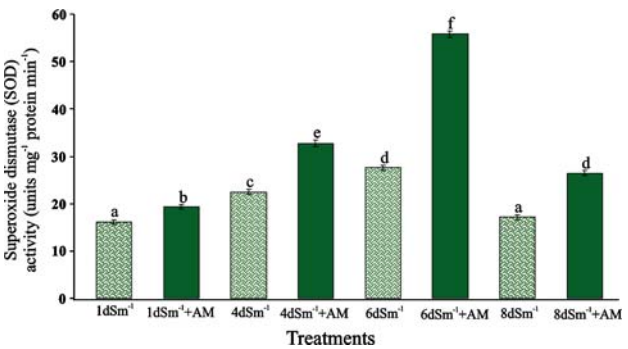

Fig. 1 Effect of AM inoculation on the superoxide dismutase (SOD) activity (units mg^{-1} protein min^{-1}) in nodules of pigeonpea under salt stress. Treatments are designed as uninoculated controls (1 dSm^{-1}), saline stress (4, 6, 8 dSm^{-1}), arbuscular mycorrhiza (AM). Data are means ± SE of six replicates. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test

levels of CAT and POX (Figures 2 and 3) activity were observed in AM plants when compared to corresponding non-AM plants subjected to moderate to heavy salt stress. The presence of AM fungi in the plant roots increased POX activity by 34.23% and 45.56% at 4 and 6 dSm^{-1}, respectively, relative to unstressed controls. POX activity of stressed AM plants was 3% and 6.04% higher than the corresponding uninoculated stressed plants at 4 and 6 dSm^{-1}, respectively.

APOX activity in the nodules declined under 6 and 8 dSm^{-1} of salt stress (Figure 4). However, mycorrhizal plants maintained significantly higher APOX activity than the uninoculated stressed plants. GR activity was greatly induced by salinity stress (Figure 5). Intraradical root colonization by mycorrhizal fungi further stimulated the GR activity irrespective of the saline treatments.

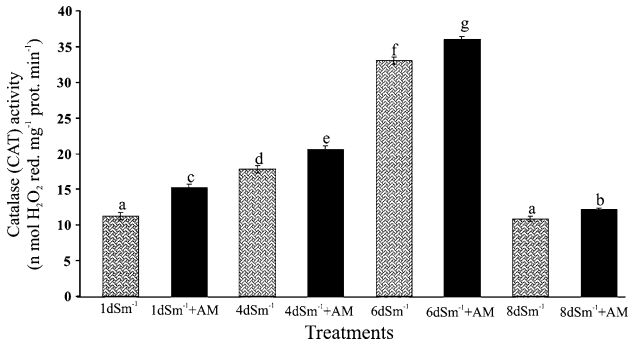

Fig. 2 Effect of AM inoculation on the catalase (CAT) activity (n mol H_2O_2 red. mg^{-1} prot. min^{-1}) in nodules of pigeonpea under salt stress. Treatments are designed as uninoculated controls (1 dSm^{-1}), saline stress (4, 6, 8 dSm^{-1}), arbuscular mycorrhiza (AM). Data are means ± SE of six replicates. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test

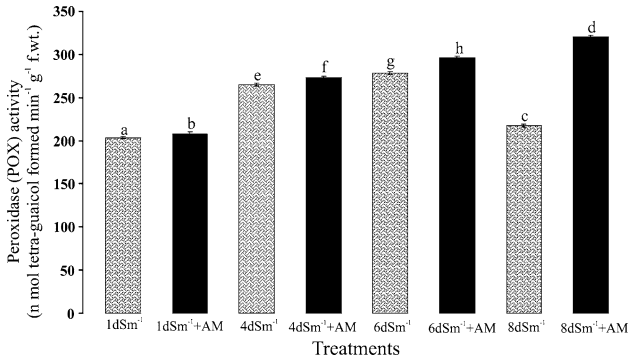

Fig. 3 Effect of AM inoculation on the peroxidase (POX) activity (n mol tetra-guaicol formed min^{-1} g^{-1} f.wt.) in nodules of pigeonpea under salt stress. Treatments are designed as uninoculated controls (1 dSm^{-1}), saline stress (4, 6, 8 dSm^{-1}), arbuscular mycorrhiza (AM). Data are means ± SE of six replicates. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test

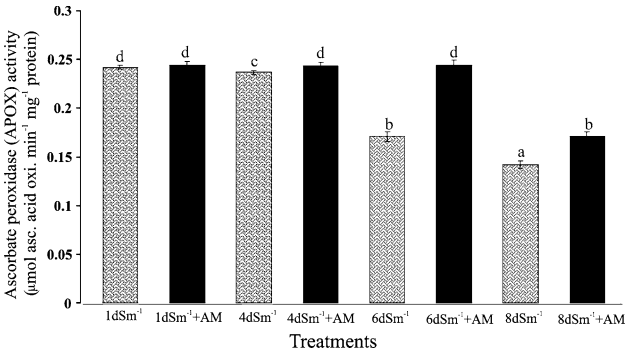

Fig. 4 Effect of AM inoculation on ascorbate peroxidase (APOX) activity (μ mol asc. acid oxi. min^{-1} mg^{-1} protein) in nodules of pigeonpea under salt stress. Treatments are designed as uninoculated controls (1 dSm^{-1}), saline stress (4, 6, 8 dSm^{-1}), arbuscular mycorrhiza (AM). Data are means ± SE of six replicates. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test

Lipid Peroxidation

The exposure of plants to salt stress leads to strong oxidation of cell components such as peroxidation of polyunsaturated fatty acids in biomembranes via free radical reactions. TBARS content (Figure 6) increased with an increase in saline concentrations in the soils. However, comparisons between non-AM and AM plants showed significantly less TBARS content in AM-inoculated plants than the non-AM plants, indicating higher lipid peroxidation in the non-AM plants under salt stress.

Discussion

The present study showed that nodule number increased at salt levels of 4 and 6 dSm^{-1} but decreased at 8 dSm^{-1} in all the plants. Nodule dry mass accumulation, nodule/shoot (NNW) ratio, leghemoglobin content, and nitrogenase activity declined with increasing salt dosages. The process of N_2 fixation was more affected by salt than nodulation. The data revealed that the reduction in dry weight under salt conditions was more closely linked to the reduction in the size of nodules rather than to the initiation of nodulated organs. Rhizobium apparently is actively infecting roots and nodules develop, but conditions within the nodules prevent them from enlarging. Rafiq (1997) and Anthraper and DuBios (2003) observed a similar increase in the nodule number under saline conditions and reported that although salt-stressed plants produced more nodules than the controls, most of the nodules were small. An increase in the average nodule weight with increasing salinity level has already been reported for chickpea (Soussi and others 1998; Garg and Singla 2004) and for faba bean (Cordovilla and others 1999).

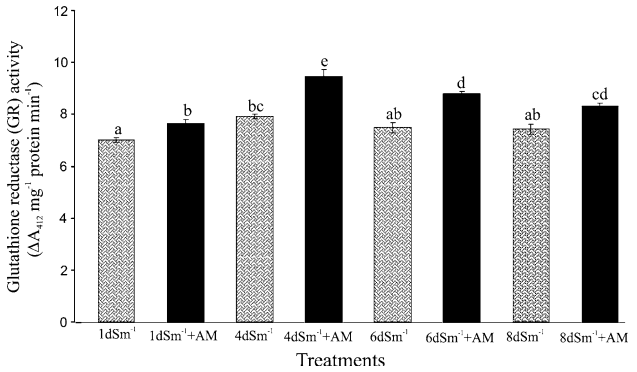

Fig. 5 Effect of AM inoculation on glutathione reductase (GR) activity (ΔA_{412} mg^{-1} protein min^{-1}) in nodules of pigeonpea under salt stress. Treatments are designed as uninoculated controls (1 dSm^{-1}), saline stress (4, 6, 8 dSm^{-1}), arbuscular mycorrhiza (AM). Data are means ± SE of six replicates. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test

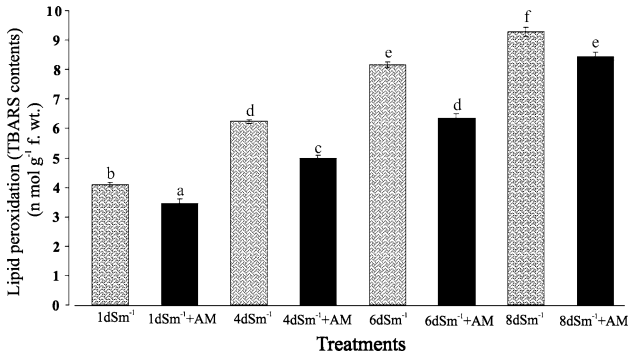

Fig. 6 Effect of AM inoculation on lipid peroxidation (TBARS contents) (n mol g^{-1} f. wt.) in nodules of pigeonpea plants under salt stress. Treatments are designed as uninoculated controls (1 dSm^{-1}), saline stress (4, 6, 8 dSm^{-1}), arbuscular mycorrhiza (AM). Data are means ± SE of six replicates. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test

Results of the present study revealed that nodule differentiation was also affected by salt, as evidenced by the appearance of brown (incompletely differentiated or senescent) nodules, which lost their pink color (that is, reduced leghemoglobin content). Acceleration of nodule greening and decline in nodule leghemoglobin expressed itself as a decline in ARA. The inhibition of nitrogenase activity under saline conditions might be attributed mainly to a physiologic effect that inhibits enzyme function. At all the salinity levels tested, the detrimental effect on nodulation and nitrogen fixation of salt was less severe on mycorrhizal-colonized plants. Among the nodular metabolic processes, reductions of bacteroid respiration and leghemoglobin production by salt have been reported as important factors that contribute to a decrease in nitrogen fixation (Delgado and others 1994). Evidence from previous studies (Johansson and others 2004; Rabie and Almadini 2005) indicates that the presence of AM fungi enhances nodulation and nitrogen fixation by legumes. It was suggested that mycorrhizal and nodule symbioses often act synergistically on infection rate, mineral nutrition, and plant growth (Patreze and Cordeiro 2004; Rabie 2005), which supports the need of both nitrogen and phosphorus and increases the tolerance of plants to salinity stress (Rabie and Almadini 2005). In the present study, AM plants showed less toxic effects of salts on nodulation compared with non-AM plants, emphasizing the prime role of AM fungi in increasing tolerance of pigeonpea plants up to 8 dSm^{-1}. Selective accumulation or exclusion of ions, control of ion uptake by roots and transport into leaves, and compartmentalization of ions at the cellular and whole-plant levels are the most effective strategies of AM fungi for adaptation of plants to salinity (Rabie and Almadini 2005).

Our results revealed that SOD activity increased with salt stress. This induction of SOD activity coincided with changes in hydrogen peroxide-scavenging enzymes (catalase, peroxidase, ascorbate peroxidase, and glutathione reductase). The increased SOD activity may result in increased H_2O_2 levels, and this is accompanied by an increased enzymatic capacity to decompose it (Hernandez and Almansa 2002). Catalase has been shown to have a positive relationship with nitrogenase activity and has been found to increase under salt stress in soybean (Comba and others 1998). A decrease in CAT activity may cause increased peroxide levels during senescence (Becana and others 1986).

In the present study, salinity stress resulted in lipid peroxidation and the TBARS content increased with increasing salt stress. Salt stress produced ion leakage, indicating injury to the membrane integrity, which could be affected by ROS formed during respiration, enhancing lipid oxidation of the membranes. Similar observations have also been reported by Lechno and others (1997), Gomez and others (1999), Savoure and others (1999), and Hernandez and others (2001).

Our results indicated that low and moderate salinity further increased SOD activity in mycorrhizal plants. The greater SOD activity in mycorrhizal plants could increase the capacity of nodules to scavenge superoxide radicals. Peroxidase activity increased with salt stress and was highest at the highest salt stress in all plants. The greater POX activity in the nodules of mycorrhizal plants suggests that POX could be involved in mycorrhizal-mediated enhancement of nodular activity under salinity. CAT activity increased with salinity in mycorrhizal plants in the nodules. This can explain better nodulation and nodule growth at higher salinities when compared to nonmycorrhizal plants. Salt stress decreased APOX

activity, however, salt stress-induced decreases in APOX activity were smaller in stressed mycorrhizal than in stressed nonmycorrhizal plants. Alguacil and others (2003) have reported that increased antioxidative enzyme activities could be involved in the beneficial effects of mycorrhizal colonization on the performance of plants grown under semiarid conditions. Our observations on the ameliorative role of AM in pigeonpea under salt stress are in line with those of Ruiz-Lozano and others (2001) and Porcel and others (2003) in soybean under drought stress.

In conclusion, the present study revealed that exposure of mycorrhizal pigeonpea plants to salinity resulted in significant induction of nodular antioxidative enzyme activities such as SOD, POX, CAT, and GR that could help the plants protect themselves from the oxidative effects of the ROS. The greater nodulation and nitrogen fixation, reduced relative permeability, lipid peroxidation, and enhanced antioxidant defense system in mycorrhizal pigeonpea plants under salt stress can be related to increased longevity of the nodules.

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