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Effects of Sodium Chloride and Mycorrhizal Infection on the Growth and Nitrogen Fixation of *Prosopis juliflora*

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Abstract

Prosopis juliflora (Swartz) DC seedlings were inoculated with a rhizobial broth (strain DUS337); half were also inoculated with arbuscular mycorrhizal (AM) fungi. Four sodium chloride (NaCl) treatments (0, 0.15, 0.3, and 0.6 M NaCl) were applied weekly, from 24 hrs after inoculation until the time of harvesting. The experiment ran for 6 weeks. Growth, nodulation, N_2 fixation and AM infection were measured. As NaCl concentration increased, plant dry mass, nodule dry mass and AM infection decreased. Nodulation ceased between 0.3 and 0.6 M NaCl, and AM infection between 0.15 and 0.3 M NaCl. Without NaCl, nodule dry mass was 56% greater in AM plants than non-AM plants. Nitrogen fixation, measured by the different techniques of ^{15}N dilution, acetylene reduction and total N content, showed comparable results. Overall N_2 fixation showed a similar pattern to that of nodulation. $\delta^{13}C$ values became less negative with increasing NaCl concentration, indicating a possible increase in water-use efficiency of plants grown in high NaCl concentrations.

Keywords: *Prosopis juliflora*, nodule, nitrogen fixation, salinity, mycorrhiza, *Glomus*, *Rhizobium*

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1. Introduction

Arid and semi-arid lands occupy about one-third of the world's surface (Dregne, 1989). In these regions, the constraints for plant growth created by limited availability of water are frequently exacerbated by low nutrient availability (Sanchez, 1987) and salinity (Valenti et al., 1991). Despite these unfavorable conditions population growth is placing pressure on these areas for crop production. With increasing interest in the development of sustainable, low input land-use systems in the tropics, agroforestry is being widely considered as an option, and N_2 fixing tree species are often planted as components of these systems (Sanchez, 1987).

The family of Leguminosae contains many economically important plant species which occur in a great diversity of habitats. Woody genera of the sub-family Mimosoideae, such as *Acacia* and *Prosopis* are frequently dominant in arid and semi-arid environments (Felker and Clark, 1980) and both genera have a potential for planting in saline soils (Rhodes and Felker, 1988; Craig et al., 1991). *Prosopis juliflora* (Swartz) DC is an important crop in East Africa and elsewhere, being used for fuelwood, charcoal and fodder (Brewbaker, 1987).

Like many other legumes, *Prosopis* not only forms associations with *Rhizobium* but also with AM fungi. This tripartite association is generally more effective than an association between the plant and only one of its symbionts (Bagyaraj, 1984). When a legume infected with both symbionts is grown in soil severely limiting in available P and N, growth enhancement is observed due both to the increased uptake of P by AM fungi and to atmospheric N_2 fixation in the root nodules (Brown et al., 1988). In addition to effects on plant nutrition, AM fungi have also been implicated in aiding water uptake (Safir et al., 1971). While the tripartite association of *Prosopis* seems to have received little attention, studies of other legume trees indicate the importance of considering both micro-symbionts (Dela Cruz et al., 1988; Manjunath et al., 1984). One such study with *L. leucocephala* (Punj and Gupta, 1988) demonstrated that inoculation with either microsymbiont improved plant growth, nodulation, and increases in nitrogenase and nitrate-reductase activities and N and P concentrations.

There are two main problems facing plants growing in saline soils: the high concentration of salts in the soil solution produces a high osmotic concentration (and a correspondingly low soil water potential), and ions such as Cl^- or Na^+ are present in high (possibly toxic) concentrations or in unfavorable combinations (usually a high sodium:calcium ratio). Plants tend to cope with these problems either by excluding salts (which removes the problem of ion toxicity but increases the problem of water deficiency) or tolerating salts (which can lead to ion toxicity and imbalance) (Greenway and Munns, 1980). It

is believed that plant growth is inhibited in salt-sensitive species due to ion toxicity. In general, agricultural legumes are thought to be salt excluders (Lauchli, 1984).

The study of the interactions between the components of the tripartite association and their interactions with their environment may help in understanding the success of *P. juliflora* in arid and saline conditions. This paper reports a study on the effect of NaCl and AM infection on growth, nodulation and N_2-fixation of *P. juliflora*.

2. Materials and Methods

Experimental design

A 2 × 4 factorial experimental design was used, testing the effects of the presence or absence of mycorrhizas (two levels) and NaCl (four levels) upon plants which were all inoculated with a *Rhizobium* strain, DUS337. Preliminary experiments (results not presented) using plants infected with both microsymbionts enabled us to best determine which levels of NaCl to use with regard to microsymbiont infection and plant survival. The AM inoculum was a mixed culture which originated from *Prosopis juliflora* growing in Kenya, and was obtained from ITE, Edinburgh, Scotland and maintained on maize (*Zea mays* L.) plants. At harvest >95% of spores resembled *Glomus aggregatum* Schenk and Smith (Baker, 1992). The experiment was laid out in six randomised locks with one replicate per treatment per block.

Germination and growth conditions

P. juliflora seeds were supplied by the Oxford Forestry Institute, Oxford, England and stored open to air at room temperature. Seeds were collected from 10 trees (part of a natural deciduous forest) in the upper part of the Comayagua Valley, Honduras, during February/March 1983. Before planting, seeds weighing 39±1 mg were surface sterilized in absolute alcohol for 1 min, followed by 1.8 M hydrogen peroxide for 7 min, then rinsed with sterile distilled water. They were scarified and germinated in a 4 l tray, filled with a mixture of water-washed and sterilised sand/vermiculite (1:1, v:v). To ensure adequate N_2-fixation it was necessary to grow plants without soil N and so a sand/vermiculite mixture was adopted.

Seedlings were transplanted 7 days later to individual pots (0.57 l) and inoculated with the appropriate symbiont. All seedlings were given a mixture of 2 g sand/rhizobial broth (5:1, by volume containing 5×10^5 cells ml^{-1} broth)

and half were also given 0.75 g (fresh mass) AM maize roots (58% of root infected with AM fungi) which were applied in a layer before planting, at a depth of 50 mm. Once a week, 20 ml of nutrient solution (Baker, 1992) containing 41.6 mM KNO_3 (5% of which was enriched with ^{15}N), 6.8 mM KH_2PO_4 and 0.7 mM K_2HPO_4 was added to each pot. Plants were grown in a growth cabinet (27±2°C) with a photoperiod 16:8 (light:dark), and a photosynthetic photon flux density of 200 μmol $m^{-2}s^{-1}$ (measured in the waveband 400–700 nm photosynthetically active radiation).

Twenty four hours after inoculation of the seedlings, the salt treatments commenced: 200 ml of the appropriate salt solution (either 0, 0.15, 0.3, or 0.6 M NaCl) was poured into the pot and the excess allowed to drain. Pots were watered each day with distilled water to maintain the potting mixture at near field capacity. Each pot was rinsed weekly with the appropriate salt solution as described above.

Plant analysis

Stem height was recorded each week. After six weeks all plants were assayed for acetylene reduction (Turner and Gibson, 1980) and 24 hours later destructively harvested. Root samples were taken for AM analysis and plant parts (shoots, roots, and nodules) were placed in paper bags and dried at 70°C until a constant mass (approximately 36 hrs). Dry masses were measured and samples taken for determination of percentage N, percentage enriched N and also $\delta^{13}C$ ratios.

Acetylene reduction assay

For the acetylene reduction assay, plants and pots were placed in a sealed plastic bag of known volume. Acetylene was added so that it accounted for 10% of the bag volume. Samples of 0.25 ml of gas were removed from the bag at 10 and 40 min after exposure to acetylene. Ethylene concentrations were measured using a Varian Aerograph series 1200 gas chromatograph with a 1,200 × 1.6 mm Porapak T (80/100 mesh) column. Gas samples were injected directly into the gas chromatograph and analysis was completed within one minute. Ethylene production was calculated as the difference between the two samples taken at different times.

Mycorrhizal analysis

At harvest, nodules were counted and separated from the root system. To allow for both AM assessment and measurement of dry mass, root systems were divided into two equal parts and the fresh mass recorded. One part was then placed in 50% (v:v) alcohol and kept for AM assessment; the other half was retained for dry mass measurements. Root dry masses were corrected for the amount used in the AM assessments. Roots for AM analysis were cleared and stained according to Koske and Gemma (1989) and root infection was determined (Giovanetti and Mosse, 1980) using the gridline intersect method.

Mass spectrometry

For C and N analysis, dried plant material was ground finely in a Glen Creston MM2 mixer mill, placed in an oven (70°C) overnight and stored in a desiccator. Samples were weighed in duplicate (0.5 to 0.8 mg per sample) on a Cahn micro-balance and used for both C and N analysis using a Carlo Erba Elemental Analyser (model 1106). The standard used was atropine (4.84% N and 70.56% C). The total amount of N in each sample was estimated from the percentage values obtained from the machine.

For mass spectrometry, plant material was finely ground as for C-N analysis, and samples (0.95 to 1.05 mg) were weighed on a Cahn micro-balance again in duplicate. Delta ^{13}C and ^{15}N values were determined using a Europa-Scientific system which measured the mass/charge ratio of the gaseous samples produced by a Robo Prep-CN biological sample converter linked to a Tracer Mass spectrometer. The reference material used was a secondary standard related to Pee-Dee belemite. The amount of N_2 fixed was calculated from the amount of isotope (5% atom % excess $^{15}NO_3^-$) dilution minus the seed N content. The amount of nitrate taken up from the soil was estimated by multiplying the % enrichment by 20 and then multiplying this figure by total N (mg). For both C-N analysis and mass spectrometry replicates of roots within treatments were pooled as were nodule replicates.

3. Results

There was a significant ($P < 0.05$) treatment effect upon plant survival; most deaths occurred at 0.3 and 0.6 M NaCl among plants which had not been inoculated with AM fungi. In total 5 deaths occurred in uninoculated plants compared with only one death in inoculated plants. All these deaths occurred during the first 3 weeks of the experiment. The remaining plants grew and were

healthy throughout the experiment. Differences in plant height between NaCl treatments became rapidly apparent. At harvest, there was a main effect of NaCl ($P < 0.001$) which led to substantial reductions in plant height and shoot and root dry mass as NaCl concentration increased (Table 1).

Table 1. Effects of NaCl upon growth of *Prosopis juliflora* seedlings, 6 weeks after the start of the experiment.

	NaCl			
Parameter	0 M	0.15 M	0.3 M	0.6 M
Height, mm	108.3 a	90.3 b	63.5 c	29.7 d
Shoot dry mass, mg	248.0 a	214.5 a	157.7 b	70.8 c
Root dry mass, mg	78.8 a	56.5 b	47.6 b	14.6 c

Means were calculated on a plant basis, for each parameter mean values (12 replicates) not sharing a letter in common are significantly different ($P < 0.001$).

In contrast with the treatment effects on height and dry mass, there was a significant interaction between increasing NaCl concentration and AM colonisation on nodule dry mass (Table 2). Although there was an overall decline in total nodule dry mass per plant with increasing NaCl concentration, there was also an effect of inoculation at 0 M NaCl in which nodule dry mass was 56% greater in AM plants than non-AM plants (Table 2). The mean nodule dry mass (total nodule dry mass/number of nodules) was also adversely affected by increasing NaCl concentration ($P< 0.001$), but there was no interaction with AM inoculation. No AM infection was detected in the uninoculated treatments. In the treatments which had been inoculated with AM fungi, infection did not occur at 0.3 and 0.6 M NaCl, and at 0 and 0.15 M NaCl there was significantly more infection (31% of the root length) in the former than in the latter (22%). Relationships between plant growth, NaCl and AM inoculation were also examined by linear regression. Regressions between height, total dry mass and shoot dry mass against increasing NaCl concentration were significant ($P < 0.001$) for both AM and non-AM plants.

Table 2. Effects of NaCl and AM inoculation upon nodule dry mass (mg) per plant, 6 weeks after the start of the experiment. Plants at 0.3 and 0.6 M NaCl that were inoculated with AM fungi were not observed to be mycorrhizal.

	NaCl			
Treatment	0 M	0.15 M	0.3 M	0.6 M
Inoculated with AM	25.3 a	21.8 ab	9.2 d	0 e
Not inoculated	16.2 b	20.8 ab	10.6 cd	0 e

Means were calculated on a plant basis, mean values (6 replicates) not sharing a letter in common are significantly different ($P < 0.05$).

Nitrogen fixation was estimated by total N determination (Fig. 1) and these results were confirmed by ^{15}N dilution and acetylene reduction. As NaCl was increased, acetylene reduction activity decreased from 168.7 μmol C_2H_4 g^{-1} dw nodule hr^{-1} at 0 M NaCl to 39.5 μmol C_2H_4 g^{-1} dw nodule hr^{-1} at 0.3 M NaCl ($P < 0.001$). No activity was recorded in plants treated at 0.6 M; these plants were not nodulated. There was no difference in activity in either AM or non-AM plants. Nitrogen accumulated from N_2-fixation was calculated from the differences between total plant N and the amount accumulated from the seed and soil nitrate. The amount of enriched nitrate taken up by the plant from the soil (Fig. 1) was similar for 0, 0.15, and 0.3 M NaCl, but decreased significantly at 0.6 M NaCl. Like the acetylene reduction values, the amount of N_2-fixation determined by the ^{15}N dilution method (Fig. 1) showed a steady decline with increasing concentration of NaCl. As samples within a treatment for both roots and nodules were pooled, statistical analysis was carried out only on shoot data which confirms that shoot N content decreased significantly with increasing NaCl concentration. Pooled data from roots and nodules showed similar results.

There was a significant interaction ($P < 0.01$) between NaCl and AM inoculation on shoot $\delta^{13}C$ values (Table 3). Overall, these values became less negative with increasing NaCl concentration and at 0 and 0.15 M NaCl, AM plants were more negative than non-AM plants. In all treatments, shoot $\delta^{13}C$ was less than root and nodule $\delta^{13}C$. The mean values of root and nodule $\delta^{13}C$ showed a similar pattern to shoot $\delta^{13}C$ values, becoming less negative with increasing NaCl concentration.

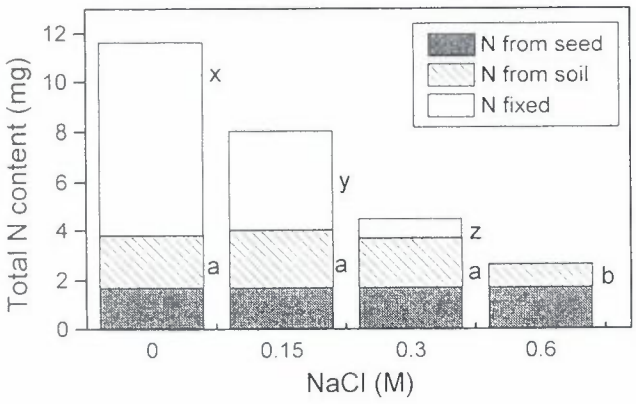

Figure 1. Effect of NaCl on the total N content (mg) of *Prosopis juliflora* seedlings, 6 weeks after the start of the experiment. Means were calculated on a plant basis, for each parameter mean values not sharing a letter in common are significantly different (P> 0.001).

Table 3. Effects of NaCl and AM inoculation upon shoot $\delta^{13}C$ values, 6 weeks after the start of the experiment. Plants at 0.3 and 0.6 M NaCl that were inoculated with AM fungi were not observed to be mycorrhizal.

	NaCl			
Treatment	0 M	0.15 M	0.3 M	0.6 M
Inoculated with AM	–32.26 e	–31.24 d	–28.86 b	–26.95 a
Not inoculated	–31.31 d	–30.19 c	–29.25 b	–26.72 a

Means were calculated on a plant basis, mean values (6 replicates) not sharing a letter in common are significantly different (P < 0.012).

4. Discussion

It has been well documented that *Prosopis* species have a high tolerance to salinity (Felker et al., 1981; Valenti et al., 1991; Valenti et al., 1992) and that certain species such as *P. cineraria* are able to grow through a salt crust.

Therefore, it was not surprising that *P. juliflora* was able not only to survive, but also to grow in the soil with the highest NaCl concentration used in this study. There was a negative relationship between plant height (and dry mass) and increasing NaCl concentration. Singleton and Bohlool (1983a) found a similar result with nodule formation and nitrogenase activity in *Glycine max* (L.) Merr.

There have been many reports that legumes infected with both rhizobia and AM fungi have a higher yield, increased nodulation and increased acetylene reduction rates (Barea et al., 1992). At 0 M NaCl we found that there was significantly more nodulation and a tendency towards increased dry mass, increased acetylene reduction and also increased N accumulation when AM fungi were present. Mycorrhizal infection, where it occurred, was at a fairly low level and hence it was not unexpected that AM effects were limited. However, it should be noted that P was freely available and this may have influenced the ability of inoculated plants to express any benefits (Bolan, 1991). Furthermore, it has been reported that N_2-fixation can be limited by lack of P (Becker et al., 1991), and that some AM fungi are more effective than others when in combination with rhizobia (Dela Cruz et al., 1988; Ianson and Linderman, 1991). With the addition of NaCl, the benefit of AM fungi to nodule development was reduced and as the concentration increased above 0.15 M NaCl, AM infection ceased. This effect of salinity on AM infection has been reported before (see Juniper and Abbott, 1993).

There are several reports that salinity adversely affects nodule dry mass and nitrogenase activity (Lauter et al., 1981; Yousef and Sprent, 1983; Marcar et al, 1991). In this study similar results were found. However, due to the relative tolerance of *Prosopis* species to NaCl and other salts, much larger concentrations of NaCl were used. Even in these high concentrations of NaCl (0.3 M), nodules still formed and expressed nitrogenase activity. In other studies (Singleton and Bohlool, 1983b; Cordovilla et al., 1994), symbionts were generally not subjected to a concentration over 0.2 M NaCl. Barea et al. (1987) reported that AM fungi improved symbiotic N_2-fixation and enhanced N uptake from the soil. In this study, there was no difference in the amount of N taken up from the soil between AM and non-AM plants, although this may have been because N was added as nitrate (Barea et al., 1987).

The acetylene reduction assay in a closed system has been criticised by Witty and Minchin (1988), however, used as an indicator that active nitrogenase is present, rather than as a quantitative assay, the method is valid. In this study, acetylene reduction rates were variable, although in general rates decreased with increasing NaCl concentration. There were no nodules present at 0.6 M NaCl and there was no acetylene reduction at this concentration. This

suggests that the bacteria or the bacteria/plant association was more sensitive to high salt than the plant itself.

Mahmood and Mahmood (1989) found similar results in that the total N content of *P. juliflora* declined as NaCl concentration increased. From preliminary studies (Baker, 1992), the ash content of *P. juliflora* increased from 9% at 0 M NaCl to 15% at 0.6 M NaCl. As this is such a small increase in percentage ash, compared with the increase in concentration of NaCl, it indicates that *P. juliflora* is largely a salt excluder. This would support results for nodule formation and rates of N_2-fixation at 0.15 and 0.3 M NaCl.

In an unlimited CO_2 supply, discrimination against ^{13}C will be high, producing a low (more negative) $\delta^{13}C$ ratio. The range of $\delta^{13}C$ ratios for C_3 plants is between –36‰ and –23‰ (Griffiths, 1991). In this study, a range of 5‰ was observed in one species, becoming less negative with increasing NaCl concentration. This suggests that the water use efficiency of *P. juliflora* effectively increases with increasing NaCl concentration. Neales et al. (1983) have also reported that $\delta^{13}C$ ratios become less negative as the salinity gradient increases, and this has been attributed to stomatal closure (Downton, 1977; Seemann and Critchley, 1985). As more stomata close, the internal concentration of CO_2 in the leaf will decrease. As a consequence of this, there is less discrimination against ^{13}C.

Interestingly, shoot $\delta^{13}C$ ratios were always less negative than the associated value for roots or nodules. There was a difference in $\delta^{13}C$ ratios between AM and non-AM plants at 0 and 0.15 M NaCl, with AM plants having a more negative $\delta^{13}C$ ratio than non-AM plants, suggesting a lower water use efficiency in AM plants. Handley et al. (1993) also found a lower $\delta^{13}C$ ratio in AM inoculated *Ricinus communis* plants and credited this to improved water status of the plant.

This work supports the view that Prosopis species have a high tolerance to NaCl. In conclusion, as NaCl increased, plants were generally smaller and infection with both symbionts eventually ceased, however, functioning nodules were still present at 0.3 M NaCl showing a large tolerance to high NaCl concentrations. The water use efficiency of *P. juliflora* appeared to increase with increasing NaCl as is supported by higher $\delta^{13}C$ values and less uptake of KNO_3 from the soil. AM inoculation would appear to benefit the plant by increased plant survival and nodulation and also by improving the overall water use efficiency. Opportunities exist in rhizobial/AM research to select and/or screen for tolerance to and effectiveness in saline soils although these were not investigated in this study. *P. juliflora* together with its symbionts has great potential for use not only in soils of low nutrient availability, but also of high NaCl.

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