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Effect of Phosphorus, Salinity and Moisture on VAM Fungal Association in Neem (*Azadirachta indica* Linn.)

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Abstract

Different strains of VAM fungi were screened for their tolerance to varying levels of P, salinity and soil moisture while infecting neem (*Azadirachta indica*). The maximum percent root infection (79%) occurred at 30 mg kg^{-1} P with *Glomus fasciculatum*, which also resulted in the highest biomass production. With increased salinity level up to $3dSm^{-1}$, there was a slight decrease in percent root infection by VAM fungi. However, when the salinity levels were further increased up to $6dSm^{-1}$, a drastic decrease (nearly one third) in percent root colonization occurred. *Glomus mosseae* was found to be the most saline-resistant species as compared to *Glomus fasciculatum*, *Gigaspora margarita* and mixed inocula. It was observed that VAM infection increased with increasing moisture stress levels. The maximum infection was observed when soil was maintained at 30–60% available water, irrespective of fungal species. *Glomus fasciculatum* sporulated the most and stimulated the highest biomass production, and appeared to be the most efficient VAM fungus for neem and therefore is recommended for propagation of neem seedlings in the nursery.

Keywords: Phosphorus, salinity, available water, VAM fungi, neem

1. Introduction

Neem (*Azadirachta indica* Linn) is considered to be an excellent tree species for shelterbelts, adaptability to diverse habitats and climatic conditions and qualifies to be an important tree species to store chemicals of multiple use. It is

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also used in pest control, toiletries, cosmetics, pharmaceutical, plant and animal nutrition and energy generation (Swaminathan, 1993). Mycorrhizae have beneficial effects on plants during times of stress. The VAM infections of neem tree were observed on roots to 250 cm depth (Bala et al., 1989). During stress, it was observed that the reproductive phenologies are delayed in the tree component of agroforestry. VAM fungi can reduce the impact of environmental stresses as drought (Sylvia and Williams, 1992) and salinity (Ruiz-Lozano et al., 1996). The leaf mortality was reduced in trees with mycorrhizae under drought stress (Bethlenfalvay et al., 1983). VAM infection has been reported to increase nutrient uptake in stressed plants (Busse and Ellis, 1985; Linderman, 1992), lower stomatal resistance (Stahl and Smith, 1984), enable plant to use water more efficiently (Sieverding, 1983; Auge, 2001), and to increase root hydraulic conductivity (Graham and Syvertson, 1984).

Information on the growth and reproduction of VA mycorrhizal fungi under saline conditions is scarce and often circumstantial (Juniper and Abbott, 1992). VA mycorrhizae have been shown to decrease yield losses of plants in saline soils (Ojula et al., 1983; Pfeiffer and Bloss, 1988; Cantrell and Linderman, 2001). This may be due to subsequent dilution of toxic ion effects, to an amelioration of osmotic stress of the plant to increased P uptake, or to some combination of these effects. VAM fungi have been shown by several workers to occur naturally in saline environments (Rozema et al., 1986; Ho, 1987) despite the comparatively low mycorrhizal affinity of many halophytic plants (Brundrett, 1991). Soil salinity may influence the growth and activity of VAM fungi via several mechanisms, either directly or indirectly (Juniper and Abbott, 1992).

An increase in the concentration of phosphorus in plants is the most often described response to VAM fungi (Marschner and Dell, 1994). Although VAM associations have been shown to stimulate photosynthesis in well-watered plants (Azcon et al., 1992), it is generally assured that the beneficial effects of VAM fungi on the host plants are a result of increased P uptake (Fitter, 1991). Katiyar et al. (1995) reported that *Morus alba* plants inoculated with *Glomus mosseae* and fertilized with 30 kg P per hectare gave similar values for plant growth, leaf yield and leaf chemical constituents to the uninoculated plants that received 120 kg P per hectare. P^{32} measurements indicated that the P was concentrated in arbuscules, compared to lower levels of P in adjacent plant cells (Schoecknecht and Hattingh, 1976).

The objective of the present study was to evaluate the different strains of VAM fungi for their tolerance to varying moisture, P levels and salinity conditions while infecting neem plants.

2. Materials and Methods

The seeds of *Azadirachta indica* were collected from a tree located at

Central Arid Zone Research Institute, Jodhpur. The seeds were graded and those of similar size were selected and soaked in water for depulping. The seeds were sterilized with 1% alcohol for 2 minutes, and the pregerminated seeds were grown for a period of two weeks in trays containing autoclaved sterilized soils. After germination the seedlings were transplanted into polythene bags containing 1 kg of sterilized soils of different treatments. One seedling was planted into each polythene bag containing nursery soils (pH: 7.2; Organic matter: 0.57%; EC: 0.1 dSm^{-1}; Olsen P: 3 mg kg^{-1}). Inoculation of neem seedlings was done by placing the inoculum (approximately 1000 infective spores kg^{-1} soils) in a layer 5 cm under the seedling soil in the polythene bags, so that the roots penetrated the inoculum as they grew, according to Jackson et al. (1972). The spores were counted by the method of Tarafdar and Marschner (1994). Inocula of *Gigaspora margarita* (AM-1005), and *Glomus mosseae* (AM-1006) were procured from the Center for Mycorrhizal Culture Collection (CMCC), TERI, New Delhi, and *Glomus fasciculatum* (Thaxter sensu Gerdemann) was procured from CAS Bangalore and tested for viable spores in our laboratory. The mycorrhizal fungi used were propagated for 8 weeks on maize grown in a greenhouse.

Effect of phosphorus

Treatments were comprised of six levels of phosphorus and five VAM fungal inoculations. Phosphorus was applied to each pot as NaH_2PO_4 at equivalent to 0 (control), 15, 30, 45, 60 and 90 mg/kg in soil. The mycorrhizal inoculation treatments were control soil, inoculated with *Gigaspora margarita*, *Glomus fasciculatum* or *Glomus mosseae*, and indigenous VAM inoculum. The indigenous VAM inoculum was the native mixed population (31% *Glomus mosseae*, 18% *Glomus fasciculatum*, 10% *Glomus albidum*, 9% *Gigaspora albidum*, 9% *Gigaspora margarita*, 8% *Gigaspora candida*, 7% *Glomus aggregatum*, 5% *Sclerocystis microcarpus* and 3% *Sclerocystis coccogena*), which was propagating in our greenhouse on *Cenchrus ciliaris* grass. *C. ciliaris* is a perennial grass and considered to be a very good host for VAM multiplication under arid conditions. There were four replications in each treatment. Watering was done on alternate days. After 4 months, plants were harvested and observations taken.

Salinity level

A similar experiment was conducted with six salinity levels (0.1 dSm^{-1} control soil, 1 dSm^{-1}, 2 dSm^{-1}, 3 dSm^{-1}, 4 dSm^{-1} and 6 dSm^{-1}) and the four VAM fungal isolates as described earlier. Sterilized soil was treated with the

calculated amounts of NaCl and $CaCl_2$ (2:1 ratio) to simulate different salinity levels. To determine the amounts of salts for developing different salinity levels, the known amount of soil was treated with varying amounts of these salts and equilibrated in three cycles of drying and wetting phases in the laboratory in a preliminary experiment. Accordingly, the calculated quantities of salt were added and mixed thoroughly in the polybag soil, and inoculation was done as described earlier. The plants were grown for four months and measurements were taken.

Effect of moisture

In this experiment treatments were of four levels of moisture (W1: 90–100%, W2: 60–90%, W3: 30–60% and W4: 5–30% of available moisture) and the four VAM treatments as described earlier. After seedling establishment (3 weeks), moisture stress treatments were applied. Different levels of water stress were maintained periodically by recording initial soil moisture contents and adding the balance amount of lost water on a weight basis. In W1 treatment, watering was done when available water (AW) dropped to 90%, in W2 from 90 to 60%, in W3 from 60 to 30% and in W4 from 30 to 5%. After a period of 4 months the plants were harvested and measurements were taken.

Root colonization

The roots were separated from collected soil samples and suitably processed to investigate the signs of infection by VAM fungi: development of vesicles, arbuscles, spores and hyphae. The root samples were washed carefully and placed in a glass vial of FAA (30 ml formalin + 5 ml acetic acid +200 ml 75% alcohol).

Further study was carried out by cutting root segments into one cm length, placing them in a glass beaker with 10% KOH solution, and boiling them for 10 minutes in a water bath. The KOH solution clears host cytoplasm and nuclei, and readily allows stain penetration. The KOH solution was drained and the samples were washed with distilled water until the brown colour disappeared. Alkaline H_2O_2 was added to the samples for 10 minutes or until the roots were bleached. The samples were again rinsed to remove H_2O_2. One per cent HCl was now added to the samples. These samples were stained in Trypan Blue following the Phillips and Hayman (1970) rapid assay of endomycorrhizal association. A total of 100-root segments were examined for each replicate and percentage of segments with colonization was calculated. The physico-chemical properties of the soils were analyzed by methods described by Jackson (1967).

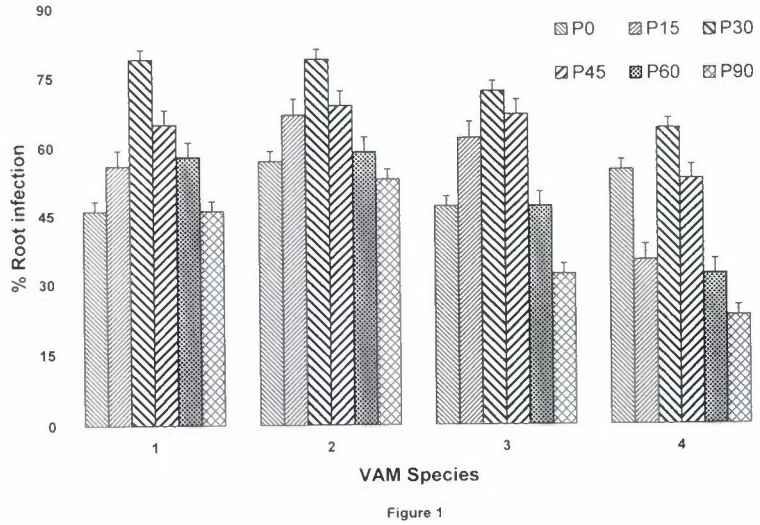

Figure 1

Figure 1. Percent root infection on 4 month-old *Azadirachta indica* by different isolates of VAM fungi under varying levels of phosphorus. 1: *Gigaspora margarita*, 2: *Glomus fasciculatum*, 3: *Glomus mosseae*, 4: Indigenous inoculum; P_0: Control soil, P_{15}: 15 mg P kg^{-1}, P_{30}: 30 mg P kg^{-1}, P_{45}: 45 mg P kg^{-1}, P_{60}: 60 mg P kg^{-1} and P_{90}: 90 mg P kg^{-1}. Vertical bars are standard errors of the differences between means.

Standard errors of means were calculated and, when appropriate, analysis of variance carried out and means of different treatments separated by the Scheffe test for planned comparisons (Sokal and Rohlf, 1981).

3. Results

Different P levels

The interaction of VAM fungi and varying levels of phosphorus showed that the greatest (79%) percent root infection occurred in 30 mg P kg^{-1} soil (P30) with *Glomus fasciculatum* (Fig. 1), whereas the least (23%) occurred in 90 mg P kg^{-1} soil with the indigenous fungal inoculum. The P_{30} level with *G. fasciculatum* was the recommended combination to achieve maximum colonization for neem seedlings.

In P deficient soil (control) the highest (57%) root colonization was observed with the *G. fasciculatum* treatment. With the *Gigaspora margarita* treatment hyphae and arbuscules were formed abundantly, but no vesicles. In contrast, *G. fasciculatum*, *G. mosseae* and indigenous inoculum treatment showed hyphae,

Table 1. Dry biomass (g/plant) of 4 month-old *Azadirachta indica* as affected by different isolates of VAM fungi under varying levels of phosphorus

Phosphorus level	S_0	S_1	S_2	S_3	S_4
P_0	2.0	3.0	3.4	2.8	3.1
P_{15}	2.3**	3.2*	3.6*	3.1**	3.1NS
P_{30}	2.8***	3.9***	4.3***	3.7***	3.6***
P_{40}	2.0NS	3.3**	4.1***	3.3***	3.0NS
P_{60}	1.8NS	2.8*	2.9***	2.6*	2.5***
P_{90}	1.8NS	2.2***	2.8***	2.3***	2.0***

S_0: Control, S_1: *Gigaspora margarita*, S_2: *Glomus fasciculatum*, S_3: *Glomus mosseae*, S_4: Indigenous inoculum; P_0: Control, P_{15}: 15 mg P kg^{-1}, P_{30}: 30 mg P kg^{-1}, P_{60}: 60 mg P kg^{-1}; P_{90}: 90 mg P kg^{-1}; Statistical significance calculated for comparison between control (P_0) and different P treatments. NS: non-significant; *p<5%; **p<1%; ***p<0.1%.

Table 2. Dry biomass (g/plant) of 4 month-old *Azadirachta indica* as affected by different isolates of VAM fungi under varying levels of salinity

Salinity level	S_0	S_1	S_2	S_3	S_4
0.1 dSm^{-1}	1.8	2.5	2.9	2.6	2.3
1 dSm^{-1}	1.6*	2.0***	2.1***	2.4*	2.0*
2 dSm^{-1}	1.5**	1.9***	1.8***	2.0***	1.7***
3 dSm^{-1}	1.2***	1.5***	1.5***	1.7***	1.5***
4 dSm^{-1}	0.9***	1.0***	1.0***	1.4***	1.1***
6 dSm^{-1}	0.7***	0.8***	0.8***	0.9***	0.8***

S_0: Control, S_1: *Gigaspora margarita*, S_2: *Glomus fasciculatum*, S_3: *Glomus mosseae*, S_4: Indigenous inoculum; Statistical significance calculated for comparison between control and salinity treatments;* p<5%; **p<1%; ***p<0.1%.

arbuscules and vesicles. No such structure was observed in the sterilized control soil. The percent infection increased with the increase in P level up to 30 mg P kg^{-1} in all cases. P rates higher than 30 mg P kg^{-1} decreased VAM colonization of neem roots.

The data recorded for dry matter production of neem as affected by VAM inoculum and different levels of phosphorus (Table 1) indicated significant increase in total biomass of plants up to P_{30} levels in all the treatments and

significant decrease in dry biomass when VAM was inoculated with P_{60} and P_{90} levels. The increase in plant dry weight in VAM-inoculated seedlings was maximum (4.3 g/pot; 54% increase over control) due to *G. fasciculatum* at P_{30} level. The study again revealed that *G. fasciculatum* was the most effective fungus followed by *Gigaspora margarita*. The effect of P levels was similar on all the VAM fungi tested.

Different salinity levels

Percent root infection in *Azadirachta indica* as affected by different VAM fungal isolates under varying levels of salinity are presented in Fig. 2. With an increase in the levels of salinity, the percent VAM colonization was reduced in all cases; the reduction was drastic above $3dSm^{-1}$ to nearly one third at $6dSm^{-1}$. *G. fasciculatum* was most affected by salinity, closely followed by *Gigaspora margarita*.

Effect of different VAM fungal isolates as influenced by varying levels of salinity on biomass of neem plants are presented (Table 2). There was a decline in dry biomass of neem seedlings with increased salinity levels. The greatest (2.9 g/pot) dry matter accumulation occurred at the control level ($0.1dSm^{-1}$) when inoculated with *G. fasciculatum*. *G. mosseae* was the most tolerant at the maximum salinity. The effect of inoculation was pronounced up to $3dSm^{-1}$ except in case of *G. mosseae* where an inoculation effect was noticed up to $4dSm^{-1}$. The trend observed in dry matter production of neem by various isolates was: *G. mosseae* > *G. fasciculatum* > indigenous inoculum > *Gigaspora margarita*. At all the salinity levels the inoculated seedlings showed more dry matter accumulation as compared to uninoculated seedlings. However, the performance of *G. mosseae* was superior in all the salinity levels as compared to other isolates.

Different moisture levels

Significant differences occurred in the percent root infection and spore production amongst various VAM fungal isolates inoculated to *Azadirachta indica* under varying levels of soil moisture stress (Fig. 3). It was observed that VAM infection increased with increasing moisture stress levels, with the maximum being in the soil maintained at 30–60% available water (AW). When soil moisture was below 30%, VAM infection decreased drastically. The highest colonization of 77% was achieved when *G. fasciculatum* was inoculated in moderately stressed plants (30–60% AW). Regardless of the growth, it was observed that the highest VAM infection and spore population occurred at 30–60% AW treatment.

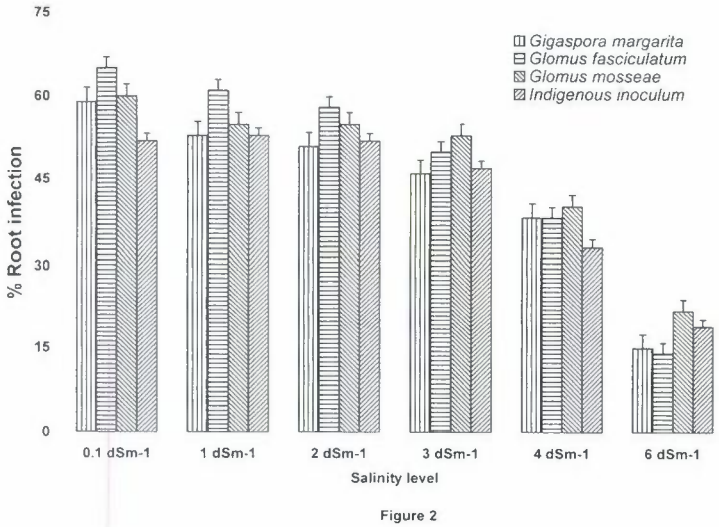

Figure 2. Percent root colonization in 4 month-old *Azadirachta indica* as affected by different isolates of VAM fungi under varying levels of salinity. 1: *Gigaspora margarita*, 2: *Glomus fasciculatum*, 3: *Glomus mosseae*, 4: Indigenous inoculum. Vertical bars are standard errors of the differences between means.

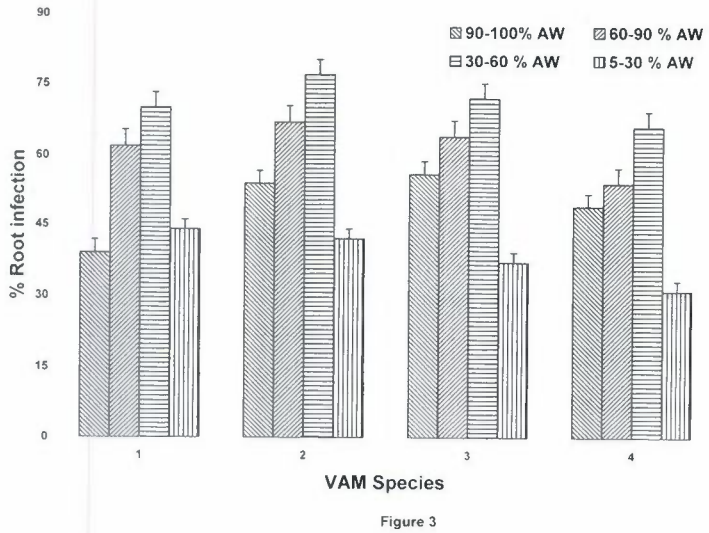

Figure 3. Percent root colonization in 4 month-old *Azadirachta indica* as affected by different isolated of VAM fungi under varying levels of moisture. 1: *Gigaspora margarita*, 2: *Glomus fasciculatum*, 3: *Glomus mosseae*, 4: Indigenous inoculum; AW: available water. Vertical bars are standard errors of the differences between means.

Table 3. Number of spores* in 4 month-old *Azadirachta indica* as affected by different isolates of VAM fungi under varying moisture levels

Available moisture (%)	S_1	S_2	S_3	S_4
90–100	211	308	232	252
60–90	408	462	307	411
30–60	650	731	589	630
5–30	313	364	281	313
LSD (p = 0.05)	12.3	7.9	8.5	6.6

*100 g^{-1} soil: S_1: *Gigaspora margarita*, S_2: *Glomus fasciculatum*, S_3: *Glomus mosseae*, S_4: Indigenous inoculum; LSD = least significant difference.

Table 4. Dry biomass (g/plant) of 4 month-old *Azadirachta indica* as affected by different isolates of VAM fungi under varying levels of moisture

Available moisture (%)	S_0	S_1	S_2	S_3	S_4
90–100	3.7	4.1	4.3	4.0	3.9
60–90	3.5	4.0	4.2	3.9	3.8
30–60	3.2	3.8	4.0	3.8	3.7
5–30	0.6	1.4	2.2	1.8	1.8
LSD (p = 0.05)	0.12	0.18	0.15	0.14	0.09

S_0: Control, S_1: *Gigaspora margarita*, S_2: *Glomus fasciculatum*, S_3: *Glomus mosseae*, S_4: Indigenous inoculum; LSD = least significant difference.

Though the dynamics of spore production varied significantly with different VAM endophytes (Table 3), a similar trend was noticed with the spore production in the rhizosphere soil. Number of spores per 100 g^{-1} soils ranged from 211 to 731. The highest number occurred with *G. fasciculatum* under all soil moisture conditions.

There was a decline in dry matter production of neem plants with the decreasing level of moisture (Table 4). The lowest in soil moisture level (5–30% AW) resulted in a drastic reduction in biomass. The data show that different fungal isolates increased the dry biomass irrespective of moisture levels with the maximum with *G. fasciculatum*. Even in severely stressed plants, *G. fasciculatum* performed better than all the other fungal isolates.

4. Discussion

The present study on neem showed that *G. fasciculatum* in combination with 30 mg P kg^{-1} soil resulted in maximum root infection and dry biomass. The beneficial response of mycorrhizal inoculation at moderate fertility may perhaps be the possible reason for the same. Better mycorrhizal colonization with inoculation may be due to uniform distribution of VAM inoculum in the soil as well as an increase in inoculum potential of *G. fasciculatum* fungi. As observed from the results, high level of P (>30 mg kg^{-1}) may decrease the functional ability of mycorrhiza as compared to its performance under moderate levels, which is in consistency with the earlier reports (Abbott and Robson, 1984; Lu and Miller, 1989). It has been suggested that rates of phosphorus application greater than those required to overcome phosphorus deficiency may induce deficiencies of micronutrients by decreasing the formation of vesicles and arbuscles (Lambert et al., 1979). It appears that neem relies moderately on VAM fungi for uptake of phosphorus, when grown in coarse textured, P deficient soil (Hedge, 1989). This may be attributed to a relatively low P buffering capacity of such soils (Rao, 1997). In P deficient soils, the phosphate nutrition of the plants is increased in presence of VAM by absorption (Mosse et al., 1973). The detrimental effect of higher doses of P on the dry matter production and percent root colonization suggested that the root colonization in neem was reduced by high soil P availability (>30 mg kg^{-1} P level).

The result from the present study indicates that high levels of salinity resulted in the decrease of VAM fungal colonization in four month-old neem plants. Nonetheless, it was observed that salt could have affect on VAM fungal spore germination (Juniper and Abbolt, 1993; Koske et al., 1996; McMillen et al., 1998)). The lower rate of infection observed in very high salinity level may also be due to the high concentration of the salts adversely affecting the endophytes, either by the high concentration of the sodium and chloride ions or by the effects of the non specific solutes on the osmotic potential. This lowering (more negative) of osmotic potential affected by the water potential, results in difficulty for the VAM fungus to take up water from the soil, or may be due to the detrimental effect of the dissolved salts in hyphal formation (Auge, 2001). Tommerup (1988) concluded that the dissolved salts in the soil solution could prevent the hyphal formation, thus preventing the VAM fungus from colonizing the host roots. The capability of VAM to decrease yield losses in high salinity environment may be attributed to decreased hyphal spread (Estaun, 1991).

Although VAM fungi mitigate growth reduction caused by soil salinity (Gupta and Krishnamurthy, 1996; Tsang and Maun, 1999), the mechanism involved remains unresolved. This may due to maintaining membrane integrity (Rinaldelli and Mancuso, 1996) that would facilitate compartmentalization

within vacuoles, and selective ion uptake. Induction of osmotica could lead to osmotic adjustment (Duke et al., 1986), and improved and balanced nutrition in plants could also increase salt tolerance (Marschner, 1995). Our study indicated that *G. mosseae* performed better in saline soil than other isolates. Ho (1987) demonstrated that *Glomus* species are well adapted to the saline environment. The performance of *Gigaspora margarita* was inferior as compared to *G. mosseae*. The reason could be the decrease in primary germ tube of *Gigaspora margarita* by high concentration of salts as has been reported by Hirrel (1981).

With the increase in the salinity level higher than $3dSm^{-1}$, a general decline in growth of inoculated neem plants were recorded. This may be due to the two distinct physiological stresses. Firstly, the toxic effect of Na and Cl, prevalent in saline soils, could disrupt the structure of enzymes, damage the cell organelles, disrupt photosynthesis and inhibit protein synthesis. Secondly, plants exposed to a high saline environment are at risk of physiological drought because they maintain lower internal osmotic potential to prevent water moving by roots into the soil (Greenway and Munns, 1980). In addition to it, higher concentration of salts may cause a decrease in the permeability of roots to water, hence lower the VAM entry to plants (Epstein, 1972). It was observed that under high levels of salinity $>3dSm^{-1}$ mycorrhizal symbiosis did not have a synergistic effect on the plant growth. This could be due to the dependency of the VAM fungus on carbohydrate nutrition by the photosynthate (Furlan and Fortin, 1977). When photosynthetic activity detoriates with high salinity level, there is a decline in the allocation of carbohydrate to the roots and therefore, the percent colonization of VAM is reduced significantly (Thomson et al., 1990).

The results of this study demonstrated that root colonization and spore production varied considerably amongst the isolates as a result of varying soil moisture stress. The differences in the number of extractable spores under varying level of moisture stress may be ascribed to the difference in the ability of VAM fungal isolates to adapt in varying soil moisture stress. In extreme conditions as high availability of moisture (90–100% AW) and severely stressed condition (5–30% AW) led to a decrease in mycorrhizal root infection and spore population. This may be due to the delayed germination of spores in severely stressed plants. VAM fungi are known to stimulate growth and to increase the drought tolerance to extreme conditions (Tarafdar, 1995; Ruiz-Lozauo and Azcon, 1995). These investigations suggest that the mycorrhizal colonization is an advantage to the host at the times of limiting moisture conditions. The mycorrhizal hyphae may act as a contact point with soil particles, and unlike root hairs, provide a longer-term mechanical link across the soil root interface. This relationship between the root surface, fungus and soil matrix would be especially important to the plants during a drought cycle.

This study indicates that the VAM isolates *G. fasciculatum* seems to have potential as field inoculum, as it is both beneficial to seedling growth and able to produce a large amount of spores and infect roots. This finding agrees with the earlier finding by Tarafdar and Praveen-Kumar (1996) in which they observed that *G. fasciculatum* was the most beneficial species in the arid environment. The fact that *G. fasciculatum* is more drought tolerant than *G. mosseae* could be attributed to the fact that *G. mosseae* spores are larger in size than *G. fasciculatum* spores (Skujins and Allen, 1986). This highlights the importance of VAM fungal colonization to drought resistance. The present results also suggest that for preparation of nursery seedlings of neem, one may use *G. fasciculatum* as inoculum so that the plants may survive in the fields under any adverse soil conditions.

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