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Research Paper

Screening of efficient arbuscular mycorrhizal fungi for *Azadirachta indica* under nursery condition: A step towards afforestation of semi-arid region of western India

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Abstract

To optimize nursery practices for efficient plant production procedures and to keep up to the ever growing demand of seedlings, identification of the most suitable species of arbuscular mycorrhizal fungi (AMF), specific for a given tree species, is clearly a necessary task. Sixty days old seedlings of Neem (*Azadirachta indica* A. Juss) raised in root trainers were inoculated with six species of AMF and a mixed inoculum (consortia) and kept in green house. Performances of the treatments on this tree species were evaluated in terms of growth parameters like plant height shoot collar diameter, biomass and phosphorous uptake capabilities. Significant and varied increase in the growth parameters and phosphorous uptake was observed for most of the AMF species against control. Consortia culture was found to be the best suited AMF treatment for *A.indica*, while *Glomus intraradices* and *Glomus mosseae* were the best performing single species cultures. It is the first time in the state of Gujarat that a wide variety of AMF species, isolated from the typical semi-arid region of western India, were tested for the best growth performance with one of the most important tree species for the concerned region.

Key words: arbuscular mycorrhizal fungi, Neem, *Glomus mosseae*, *Glomus intraradices*.

Introduction

Neem (*Azadirachta indica* A. Juss) is one of the most important tree species in the western part of the Indian subcontinent, especially in the state of Gujarat. Every year a large target is set to the Gujarat State Forest Department to produce seedlings of this tree species in abundance in the nursery to meet various objectives including a rigorous afforestation program. Attempts are often made to reclaim degraded soils and wastelands of this part of the country with these nursery raised seedlings as a part of these activities. Neem which is an important multipurpose tree species has been observed to be well suited to all kinds of lands and wide range of environment (Tewari, 1992), especially tolerant to poor soils of arid and semi arid regions (Benge, 1989) and hence makes an obvious choice for the mission.

Rapid production of high quality seedlings in nurseries is a pre-requisite for any aggressive re-forestation program, but it has been observed earlier (Michelsen, 1992) that optimal nursery conditions can hardly be achieved for quality seedling production. Moreover, there may be severe shortage in microbial populations including arbuscular mycorrhizal fungi (AMF) owing to the fact that soils used in the nursery are often collected from barren surroundings, subsoil or may have been stored for a long time (Muthukumar *et al.*, 2001). AMF is an imperative component of soil microbial biomass influencing essential processes at the plant+soil interface (Harley and Smith, 1983; Bagyaraj, 1984; Rajan *et al.*, 2000). To counteract the transplantation shock seedlings of tropical tree species raised in the nursery may need to be associated with AMF (Urgiles *et al.*, 2009) besides gaining several conventional benefits of AMF association in terms of growth and nutri-

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ent uptake. It may further be noted that *in vitro* culture of AMF is rather cumbersome and hence a large quantity of inoculum production is highly cost ineffective to warrant inoculation of the soil under field condition. Inoculation in the nursery, on the contrary, may provide dual benefits of being inexpensive and providing better vigour of seedlings to cope with transplantation.

AMF are rather obligate symbionts and their role in plant growth and nutrient uptake is well documented (Srinivas *et al.*, 1988; Arya, 1999; Sumana and Bagyaraj, 1999; Garg *et al.*, 1999; Kumar *et al.*, 2000; Bhattacharya *et al.*, 2000; Sharma and Adholeya, 2000; Prasad, 2002). It has been observed that roots of Neem are profusely colonized by AMF and it is considered as a highly mycorrhizal dependent tree species (Habte *et al.*, 1993).

Most AMF can form association with susceptible plants, regardless of the genetic diversity or geographical distribution of the two symbionts. Furthermore, the formation and function of mycorrhizas can be quite variable among fungal species and even among isolates of the same species (Smith and Smith, 1997; Smith *et al.*, 2000). A fairly wide range of functional diversity in AMF could have lead to the observed differences among fungal species in the manner they facilitate host plants in resisting biotic and abiotic stress (Smith and Read, 1997). It is, however, more conventional to define in terms of plant growth responses, which may vary monotonically from unprecedented increment in growth to neutral depending on the particular plant-fungus combinations and environmental conditions (Johnson *et al.*, 1997). Plant fungus combinations that promote optimum plant growth are believed to be those that provide the greatest amount of Pi to the plant for the least amount of photosynthate transferred to the fungus (Burleigh *et al.*, 2002). Further, it was reported that different species of AMF differ to the extent by which they increase nutrient uptake and plant growth (Bagyaraj *et al.*, 1989). Hence, the need for selecting efficient AMF that can be used for inoculating different plants was emphasized (Bagyaraj *et al.*, 1989). Most of the studies performed earlier in this context are either highly region specific or symbionts specific and knowledge base is far from sufficient as far as the western part of India is concerned.

The objective of the present study was to screen the AMF species, isolated from the semi-arid region and local AMF isolated from the rhizosphere of Neem in order to select an efficient inoculant species of AMF for Neem in nurseries.

Materials and Methods

Experimental site

The study was conducted at the forest nursery of Basan Research range, Gandhinagar, Gujarat (23°13' N: 72°41' E, altitude: 80 m). The Gandhinagar district has multi seasonal climate with an average precipitation of 667 mm. Minimum temperature records 7.5 °C in winter and maximum temperature records 45 °C in summer. [Gandhinagar District Panchayat, Gujarat Government 2008-09]. Plants were grown using a 14 h day /10 h night cycle.

Plant and fungus material

Seeds of Neem were procured from Basan research range, Forest Department of Gujarat. Seeds were collected from candidate plus trees (CPT) of Gandhinagar research range. Sand+soil based pure cultures of *Glomus intraradices, Glomus reticulatum, Glomus fasciculatun, Glomus mosseae and Glomus constrictum* were obtained from Arid Forest Research institute (AFRI), Government of India, Jodhpur, Rajasthan. Pure cultures of *Glomus aggregatum* and mixed inoculum (consortia) culture were prepared from soil samples collected from nearby plot of Neem plantation at Basan research Range. The composition of consortia was identified to be *Glomus* (79%), *Gigasspora* (8%), *Sclerocystis* (5.3%), *Scutellospora* (5%), *Acaulospora* (2.7%) following *Schenck and Perez* (1987). These fungi were multiplied as pot culture using sterilized sand:soil mix (1: 2 v/v) as the substrate and *Zea mays* as the host in a green house. After 120 days of growth, shoots of *Zea mays* were severed and the substrate containing hyphae, spores and root bits was air-dried and used as the inoculum.

Seedling production

Seeds were sown in plastic trays filled with sterilized sand: soil mixture (1:2 v/v). Sterilization was done by autoclaving the sand:soil mixture three times at 121 °C and 15 psi pressure for 60 min at an interval of one day. Seeds were germinated in the germination tray within 8 days and the seedlings were maintained there for another 3 days. At the end of 11 days the healthy seedlings of uniform length (2.5 cm) were selected and transferred to root trainers of 150 cc capacity containing unsterile nursery soil:sand:compost (2:1:1 v/v). The soil had a pH of 8.4 (1: 1 soil to water extract ratio), EC- 0.24 mS cm^{-1} and organic carbon- 0.29% (Walkley and Black, 1934). It contained 12.5 mg kg^{-1} available P (Olsen *et al.*, 1954), 180.35 mg kg^{-1} available K Ghosh *et al.*, 1983), and an indigenous AMF population of 206 spores 100 g^{-1} of soil and an inoculum potential of 900 infective propagules g^{-1} soil (Porter, 1979). The seedlings were maintained there for 75 days.

AM inoculation

Two month old seedlings were inoculated with seven AMF inocula viz. *G. intraradices, G. reticulatum, G. fasciculatum, G. mosseae, G. constrictum, G. aggregatum* and consortia. On the basis of their abundance in these soil types, emphasis was given on the *Glomus* species while selecting the AMF isolates. Each seedling in the root trainer was inoculated near the root zone at the rate of 10,000 infective propagules. Same amount of sterile inoculum,

which had been autoclaved thrice at 121 °C and 15 psi pressure for 60 min at an interval of one day, was applied to the control (non-noculated with AMF) seedlings. The experiment was laid out in completely randomized design (CRD). There were four replicates for each of the eight treatments (including control) with 24 plants per replicate, total 768 plants. Watering was done as per requirement.

After 75 days seedlings were transferred from root trainers to polybags containing 1.5 kg of same nursery soil: sand: compost mixture and maintained under ambient condition with normal watering in the green house. Each bag contained one seedling. The bags were rearranged every 15 days to ensure uniform growth conditions.

Data collection

Plant height was measured from soil surface to the growing tip of the plant. Collar diameter was measured 1 cm above the soil surface using vernier callipers. These two parameters were recorded bimonthly after plantation. However, only observations recorded at the end of the experiment are presented in this paper. Plants were harvested after 210 days. At harvest, observations on morpho-physiological parameters like, plant fresh weight, plant dry weight, root colonization, spore density and phosphorous uptake were recorded.

Plant dry weight was determined after drying the plant sample at 80 °C to a constant weight in a hot air oven.

The AM fungal spores were isolated from the root-zone soil by wet sieving and decanting technique (Gerdemann and Nicolson, 1963), examined under stereo zoom microscope (Leica® Combistereo), counted and expressed as spore density. For examining root colonization, roots of seedlings receiving different AMF treatments were separated from soil samples and processed to investigate the mode of colonization by AMF. The roots were cut into 1 cm pieces, cleared and stained with trypan blue (Phillips and Hayman, 1970). 100 root pieces were selected randomly, mounted in lactophenol, and examined for AM fungal infection by using compound microscope. The Percentage of root colonization was determined according to Giovannetti and Mosse (Giovannetti and Mosse, 1980).

Phosphorus estimation

Grinded and sieved (< 2 mm) plant samples were digested, using tri-acid mixture as described in (Jackson, 1973). 0.5 g plant samples were placed in 100 mL cooled conical flask and then 5 mL of tri-acid mixture (HNO_3: H_2SO_4: $HClO_4$), (9:2:1) was added. The digestion was carried out at 180 to 200 °C until a clear solution remained after the acids were largely volatilised. After complete digestion the final volume was made up to 50 mL. For phosphorus estimation 10 mL of digested plant material was placed in 50 mL volumetric flask, with 20 mL distilled water and 15 mL mixture of dilute HNO_3, Ammonium molybdate (5%) and Ammonium metavanadate (0.25%) in equal proportions (1:1:1). The volume was made up to 50 mL with distilled water and the yellow colour intensity was read at 485 nm using Systronics Vis Spectrophotometer-166.

Statistical analysis

The final data was subjected to one - way Analysis of Variance (ANOVA). Based on the outcome of ANOVA on all data, post-hoc analysis had been performed in the form of Duncan's Multiple Range test (Duncan, 1955) to separate the means. Linear regression analysis was used to assess the relationship between AMF colonization, growth parameters and phosphorous uptake.

Results

The response of Neem to inoculation with different AMF treatments showed certain distinctive features. Results of height, plant biomass (fresh and dry) and collar diameter are shown in Table 1. Significantly longer plant height had been observed for all the treatments against control. Plant height had increased maximally for consortia (mixed inoculum) (41.06%) against the control. Consortia

Table 1 - Effect of different treatments on growth of *A. indica*.

Treatment	Plant height* (cm)	Fresh weight* (g)	Dry weight* (g)	Collar diameter* (mm)
Control	20.19 e	49.00 ef	17.00 def	2.47 b
G. aggregatum	25.48 d	44.75 fg	17.00 de	3.22 a
G. fasciculatum	26.33 cd	47.75 efg	16.00 efg	3.57 a
G. constrictum	26.43 bcd	52.50 de	16.00 efg	3.31 a
G. reticulatum	26.45 bcd	57.25 d	18.50 d	3.47 a
G. mosseae	27.06 bc	73.00 abc	23.75 bc	3.24 a
G. intraradices	27.40 b	73.25 ab	24.75 ab	3.36 a
Consortia	28.48 a	75.25 a	26.50 a	3.39 a

*Mean of 60 observations.
Values without common letters differ significantly at p = 0.05.
Significance tested by Duncan's Multiple Range Test.

had been closely followed by *G. intraradices* (35.7%) and *G. mosseae* (34%). *G. reticulatum*, *G. constrictum* and *G. fasciculatum* were at par with each other. *G. aggregatum* and *G. fasciculatum* varied significantly with *G. intraradices* while significant variation had also been observed between *G. aggregatum* and *G. mosseae*. Collar diameter increased significantly in all AMF treated plants as compared to control. However, no significant variation had been observed among the other treatments. Collar diameter had increased maximally by AM inoculant *G. fasciculatum* (44.53%) against control. Inoculated plants exhibited significant increase in plant biomass with respect to untreated controls. Majority of the treatments except *G. aggregatum, G. fasciculatum* and *G. constrictum* had shown significant increase in plant fresh weight as compared to control. Consortia treatment had shown maximum increase (53.57%). Consortia, *G. intraradices* and *G. mosseae* varied significantly with respect to *G. reticulatum*, *G. constrictum*, *G. fasciculatum* and *G. aggregatum.* However, no significant difference had been observed among Consortia, *G. intraradices* and *G. mosseae* as well as between *G. fasciculatum* and *G. aggregatum*. With respect to dry weight, only Consortia, *G. intraradices* and *G. mosseae* varied significantly from the control. Maximum increase in plant dry weight had been observed for Consortia (56%). Performance of consortia, *G. intraradices* and *G. mosseae* are rather similar.

AMF inoculation in Neem seedlings had exhibited significant influence on uptake of phosphorous. Plants inoculated with *G. intraradices* and *G. fasciculatum*, showed significantly higher P concentration in leaves than non-inoculated control, while, for *G. reticulatum*, *G. mosseae, G. constrictum* and control similar responses had been recorded (Table 2). Minimum P concentration in leaves had been observed for *G. aggregatum*.

Microscopic examination of stained roots had shown colonization with all the species of AMF tested, as shown in Table 3. Hyphal structures were found in abundance in plant root samples. All the treatments varied significantly with the control plants both in case of root colonization and spore density. Highest root colonization percentage (97.5%) was recorded with *G. intraradices* as compared to 61% in non-inoculated control. At the end of the experiment, maximum AMF spore count was recorded for the treatment *G. mosseae* (630 100 g^{-1} of soil) showing its better proliferating ability as compared to other AMF (Table 3).

High correlation (r = 0.92) was observed between percentage root colonization and plant height. Phosphorous concentration in leaves was highly correlated (r = 0.82) with plant dry weight.

Discussion

The data acquired from nursery trial undoubtedly gave a clear evidence of positive response of Neem towards mycorrhization by AMF. This work may prove to be essential for implementation of AMF inoculations in the Gujarat state forest nurseries much more effectively and as a routine process. Inoculation with different species of AMF resulted in the improvement of growth and nutrient uptake to different extent. AMF species selected for the study were given preference on the basis of their abundance in rhizosphere soil of Neem present in arid and semi-arid soils of western India (Mohan and Verma, 1995; Pande and Tarafdar, 2004). Those AMF species were given preference for inoculation purpose in view of their higher probability of symbiotic association with Neem.

From the outcome of the trial it can be inferred that AMF inoculation in unsterile soil definitely boosted the growth of the seedlings, as previously reported from other plant species (Michelsen, 1993; Vasanthakrishna *et al.*, 1994). In general it had been observed that significant growth and nutrient uptake efficiency were obtained when the number of infective propagules and/or spore density

Table 2 - Effect of different treatments on phosphorous concentration in leaves of *A. indica*.

Treatment	*A. indica*
	Phosphorous concentration in leaves*(%)
Control	0.10 cde
Consortia	0.07 efg
G. aggregatum	0.04 g
G. constrictum	0.10 cdef
G. mosseae	0.11 cd
G. fasciculatum	0.21 ab
G. reticulatum	0.11 c
G. intraradices	0.23 a

*Mean of 4 observations.
Values without common letters differ significantly at p = 0.05.
Significance tested by Duncan's Multiple Range Test.

Table 3 - Effect of different treatments on root colonization and spore number.

Treatment	Root colonization* (%)	Spore number/100 g soil*
Control	61 c	271 d
Consortia	90 ab	562 abc
G. aggregatum	86.5 b	514 c
G. constrictum	84 b	596 ab
G. mosseae	93 ab	630 a
G. fasciculatum	88 ab	510 c
G. reticulatum	85 b	555 abc
G. intraradices	97.5 a	557 abc

* Mean of 8 observations.
Values without common letters differ significantly at p = 0.05.
Significance tested by Duncan's Multiple Range Test.

was low in the soil (Reena and Bagyaraj, 1990). However, the soil used in this study was having a considerable spore population (219 spores 100 g^{-1}). Hence, the improved effect of inoculation in the unsterilized substrate can be attributed to the ineffectiveness of the indigenous AMF, in agreement with the earlier findings (Bagyaraj *et al.*, 1989; Reena and Bagyaraj, 1990) and better competitive ability of the introduced AMF over the native AMF in colonizing the roots. Presumably the indigenous inoculum level of AMF was inadequate to support plant growth to the extent as observed in the presence of an additional mycorrhizal inoculum.

G. intraradices and *G. mosseae* were found to be the best single species cultures for Neem in terms of the growth parameters, AMF colonization and spore density. This is in contrast with the earlier findings (Prasad, 2002) where *G. fasciculatum* had been screened as the best suited species for Neem. Improved growth parameters in plants are often complemented by enriched nutritional whereabouts (Jeffries, 1987). In case of P uptake, *G. intraradices* was closely followed by *G. fasciculatum*. This also emphasizes the importance of host specificity for determining the role of AMF. A rather wide range of variation in the symbiotic capacity of all AMF inoculants with respect to this particular tree species concerned was observed. Relatively higher P concentration (0.23%) in leaves had been recorded for *G. intraradices* in our case than that recorded (0.126%) by Muthukumar *et al.* (2001), in shoot at 120 days after transplantation. In any case, mixed inoculum (consortia) showed the best performance in terms of plant height and plant biomass (Table 4). The success of a mixed inoculum depends largely on the nature of competition between endophytes. The risk involved in this case is that the competition may decrease the growth response that a single endophyte would have produced. It can be noted that several factors including soil pH, (Koomen, *et al.*, 1987), soil N and P concentration (Satir and Duniwav, 1982), soil moisture and temperature (Satir and Duniwav, 1982) may influence the rate of infection and the effect of the competitive fungi on the growth of the host plant. In the present experiment, competition among constituent species of AMF in consortia did not exhibited any decrement in growth response, instead, the particular constituent species in consortia were probably more successful in enhancing plant growth and showed better competitive ability against any other single species of AMF. This agrees with the result of Daft and Hogath (1983), who found better consistency in growth responses of plants to triple and quadruple inocula as compared to other treatments, especially under unpredicted and unsterilized soil conditions (Koomen *et al.*, 1987).

According to the results of this study, it can be noted that a definitive coherence was observed between AMF root colonization and plant growth parameters. This supports the fact that higher root colonization allows more host-fungus contact and the exchange of nutrients helps in better growth (Mallesha and Bagyaraj, 1990). AMF may

Table 4 - Treatment showing maximum growth response.

Plant height		Fresh weight		Dry weight		Collar diameter		P uptake	
Treatment showing maximum	(%) increase over control	Treatment showing maximum	(%) increase over control	Treatment showing maximum	(%) increase over control	Treatment showing maximum	(%) increase over control	Treatment showing maximum	(%) increase over control
G. intraradices	41.06	Consortia	53.57	Consortia	55.88	*G. fasciculatum*	44.53	*G. intraradices*	130

differ in their symbiotic effectiveness, which depends on their preference for particular soils or host plant specificity (Dhillon, 1992), direct ability to stimulate plant growth (Abbott and Robson, 1978; Govinda Rao *et al.*, 1983), rate of infection (Rajan *et al.*, 2000; Abbott and Robson, 1982; Miller *et al.*, 1987) and competitive ability (Michelsen, 1993). Increased P uptake in AMF treated plants was observed. This may be attributed to increased absorption through extramatrical hyphae of AMF or the enhanced growth of AMF roots may have facilitated the P absorption from soil.

From the above trial, it can be summarized that for *A. indica*, consortia, with the composition specified in this work, has performed best in terms of plant height and plant biomass followed by *G. intraradices*. However, *G. intraradices* turned out to be best in case of P uptake and root colonization. Such trials should be repeated in future to further streamline the best available growth boosting AMF species for each particular forest tree species. Thereafter such treatments with AMF can be adopted as a regular practice at the nurseries of Gujarat for raising specified forest tree species. This approach will surely help realizing the uphill task of aggressive reforestation of this semi-arid part of the country.

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