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SHORT COMMUNICATION

Mycorrhizal inoculation in neem (*Azadirachta indica*) enhances azadirachtin content in seed kernels

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Abstract In view of the high mycorrhizal dependency of neem trees (*Azadirachta indica)*, an experiment was conducted to study if Arbuscular Mycorrhizal (AM) inoculation can enhance the azadirachtin content in seed kernels of trees grown in the field. Azadirachtin is an important active ingredient in neem seed kernels based on which a large biopesticide industry has emerged in India and few countries in Europe and the USA. Inoculation of neem seedlings in the nursery with *Glomus fasciculatum* and *Glomus mosseae* resulted in increased height, dry weight, root colonization and phosphorus (P) content. In a separate experiment, field-grown neem plants inoculated in the nursery and during transplantation with *Glomus fasciculatum* were evaluated after 5 years. No significant differences were found in the tree height, girth at breast height (GBH) and fruit yield but oil percentage, total triterpenoids and azadirachtin content in kernels increased significantly as a result of AM inoculation. A similar enhancement in azadirachtin was noted with P application. These results open up possibilities of producing quality neem seed with high bioactive ingredients through AM inoculation.

Keywords AM fungi · Azadirachtin · *G. fasciculatum* · Neem

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Introduction

The neem tree (*Azadirachta indica*) has been receiving global attention recently in view of its multiple uses, more particularly, the biologically active ingredients in its seed kernels, based on which a large number of home-made and commercial biopesticide formulations are being developed all over the world (Schumutterer 1995). Neem is widely distributed in its native countries like India and Myanmar and its various products are extensively used by the village communities. Many countries in Africa and Latin America are taking up plantations of neem for checking soil erosion, as a source of biopesticide, fodder, timber and fuel wood in rural areas. Even in India, a large number of trees are planted every year by farmers, by Government under social forestry schemes and by private industries as commercial plantations because of its importance as source of raw material for the biopesticide industry. Neem has been reported as a highly mycorrhizal-dependent tree (Habte et al. 1993; Sumana and Bhagyaraj 2006). Many species of AM fungi colonize neem roots under natural conditions but the most important are *Glomus fasciculatum* and *Glomus mosseae*. Inoculation with *G. mosseae* and *G. fasciculatum* alone (Sumana and Bhagyaraj 2006) or in combination with other nitrogen-fixing and phosphate-solubilizing organisms (Muthukumar et al. 2001) improved seedling growth, quality and nutrient uptake under nursery conditions both in sterile and unsterile soils. However, no information is available so far on the effect of AM inoculation on mature neem trees, particularly on the production of bioactive compounds of economic value. Recent research showed that AM symbiosis enhances the biosynthesis of several economically useful secondary metabolites like carotenoids and terpenes in annual and perennial plants (Zhi-Lin et al. 2007). The objective of this

study was to assess the effect of AM inoculation on neem seedlings in the nursery to understand the colonization and growth promotion effect on 5-year-old adult trees in the field and to understand the effect on fruit yield, oil and azadirachtin contents.

Materials and methods

Two separate experiments were conducted to study the impact of wild strains of arbuscular mycorrhiza, both on seedlings and field grown trees. The experimental details are described below.

Seedling experiment

Four treatments viz., inoculation with *Glomus fasciculatum*, inoculation with *Glomus mosseae*, phosphorus application (20 mg kg^{-1} of P) and uninoculated control were tested with 10 replications each. These two cultures were selected based on preliminary screening of several indigenous AM fungi associated with neem. Micropropagated neem plantlets were used in this experiment in order to avoid variation if any, due to differences arising out of seed source (seed raised progeny of neem show some variability due to the occurrence of limited cross pollination). These plantlets are produced through in vitro shoot multiplication from nodal explants and ex vitro rooting in polyethylene bags filled with cocopeat in the shade house as per the protocol described by the authors (Venkateswarlu et al. 1998). AM inoculation was done after the plantlets have successfully rooted and hardened in the shade house. One gram soil-based inoculum of each species containing 50–80 spores/g was used to inoculate each of the seedlings in the polyethylene bags. After inoculation, the plants were maintained under ambient conditions in the nursery with normal watering for 3 months. At this stage, the experiment was terminated, the plants were harvested and observations on height, biomass, nutrient uptake and root colonization were recorded.

Field experiment

For field experiment, a separate set of plantlets produced as described above were used. Three treatments viz., inoculation with AM, phosphorus application and no inoculation were tested with four replications, each replication consisting of six plants. *G. fasiculatum*, was used here because of its better performance in the seedling trial. For the first treatment, plantlets already inoculated in the nursery as in the seedling experiment were transplanted (90 days after inoculation) into pits of 45 × 45 × 45 cm with a spacing of 8 m × 6 m in Hayatnagar research farm of the institute near Hyderabad, India. The experimental site was a sandy loam soil (OC, 0.5%; total N, 0.04%; available N, 128 kg/ha; available P, 9.5 kg/ha and available K, 13.0 kg/ha). During transplantation, 50 g of soil-based inoculum containing 50–80 spores/g was placed below the seedling. This way, in the field experiment, neem plantlets received inoculum twice i.e. once in the nursery and a second time during transplantation. Plantlets not inoculated during nursery and transplantation stages, served as uninoculated controls. For phosphorus application treatment, 250 g P_2O_5/plant (equivalent to 749 mgP/kg soil) was applied by uniform mixing with the soil used for refilling the pit. The pits were refilled with a mixture of original soil plus FYM in 3:1 ratio. The transplanting was done during June 2001 at the beginning of the rainy season and the plants grew entirely on rainfall received, which is the normal practice of raising neem in this region. This region receives annual rainfall of 730 mm during June–September. The plants were allowed to grow in the field for 5 years by which time all trees flowered and produced seeds. Data on tree height, girth were recorded annually while fruit yield, oil and azadirachtin contents were estimated after 5 years.

Plant growth observations

In case of the seedling trial, the total plants were harvested; root and shoot length and biomass were determined. The N, P and K content of plants (AOAC 1950), chlorophyll in leaves (Arnon 1949) and root colonization (Giovannetti and Mosse 1980) were estimated according to standard methods. For field grown plants, the height and GBH were recorded annually. Fruit yield, oil percent, triterpenoids and azadirachtin content were determined 5 years after planting.

Biochemical analysis

For determining the azadirachtin content in neem seeds, the method described by Govindachari et al. (1990) was followed which is described briefly. Neem kernels dried to 9–10% moisture were ground and extracted with petroleum ether for defatting, followed by methanol extraction. The methanol extract containing azadirachtin was concentrated by a rotary evaporator. The residue was dissolved in methanol and made up to 50 ml. An aliquot of 20 µl was injected into a Rheodyne injector. Analysis was done on a Shimadzu HPLC using RP C-18 column with acetonitrile: water (40:60) as mobile phase under a flow rate of 1 ml/min and detected with a UV detector (217 nm). The standard

azadirachtin samples (96%) supplied by Trifolio GMBH were used as reference.

For estimating total triterpenoids, the kernels were extracted with methanol, which is concentrated and poured into water. The ethanol-water mixture was extracted with hexane to remove oil and low molecular weight fatty compounds. The aqueous methanolic extract was successively extracted with ethyl acetate. Ethyl acetate extract was shaken with water and the extract was dried over anhydrous sodium sulphate to remove residual water. This ethyl acetate solution was distilled under reduced pressure. The resulting semi solid mass represented the total triterpenoids in the seeds.

Data analysis

The pooled data from the replicates of the different treatments were subjected to analysis of variance (ANOVA, $P < 0.05$) and the critical difference (C.D) between them was calculated at 5% level of probability.

Results

Seedling growth

Inoculation with both *G. mosseae* and *G. fasciculatum* significantly increased the plant height, collar diameter and dry weight of 90-day-old neem seedlings (Table 1). The differences in growth parameters were however not significant between the two AM species although *G. fasciculatum* produced higher seedling biomass over *G. mosseae*. Under uninoculated plants also, we recorded 52% root colonization, which improved to 81% with *G. fascciulatum* inoculation. Large numbers of vesicles of *G. fasciculatum* were found in the roots and in extra matricular region due to inoculation (Fig. 1). The P uptake and percent root colonization also improved significantly due to inoculation. However, the increase in N and K contents were non-significant. Total leaf chlorophyll content increased from 1.552 g fresh leaves to 1.621 and 1.728 mg respectively in *G. mosseae* and *G. fasciculatum* treated plants.

Growth, yield and biochemical parameters in field grown trees

In contrast to seedlings, we did not find significant impact of AM inoculation on the height and GBH of field grown trees when measured 5 years after transplanting when all the trees had produced measurable fruit yield. However, up to 2 years after transplantation, AM inoculated and P applied saplings recorded more height (4.1, 4.6, 4.7 m, respectively in control, AM and P applied treatments) and GBH (24, 28, 27 cm), but the differences disappeared latter. Though the plants under all treatments flowered during the fourth season (February–March, 2005), AM inoculated plants flowered about 2 weeks before the control and produced more flowers at the first flush, but the fruit yield was not significantly different. All but one plant (in the control) produced fruits during the fourth season (June–July, 2005) but there was high variability and some individual trees yielded very small quantity. During the next season (May–June, 2006) all trees produced significantly higher fruit yields than in 2005 and there was more uniform bearing and yield among trees (hence data is presented only for year 5). Though AM-inoculated and P-applied trees produced marginally higher air-dried fruit yield, the differences were not statistically significant (Table 2). However, seeds from trees inoculated with AM had significantly higher oil percentage, total triterpenoid and azadirachtin contents. A similar effect was found with P application. The overall data revealed that both AM inoculation and P application significantly enhanced the triterpenoids and azadirachtin contents in seed kernels of field-grown neem, though tree growth itself was not significantly improved.

Discussion

In the present study, we clearly observed a growth enhancement effect of AM (*G. fasciculatum)* on neem at

Table 1 Height, collar diameter, biomass, P-content, root colonization of neem sapling inoculated with AM in nursery

Treatment	Plant height (cm)	Collar dia (mm)	Seedling biomass (g/plant)	Shoot P content (mg/plant)	% Root colonization
Uninoculated	13.5	3.1	3.8	3.10	52
G. mosseae	18.2	3.9	5.6	6.72	75
G. fasciculatum	19.6	4.2	6.0	6.60	81
Phosphorus applied (20 mg kg^{-1})	17.6	3.7	4.9	6.37	62
CD at 5%	0.95	NS	0.45	0.37	3.00

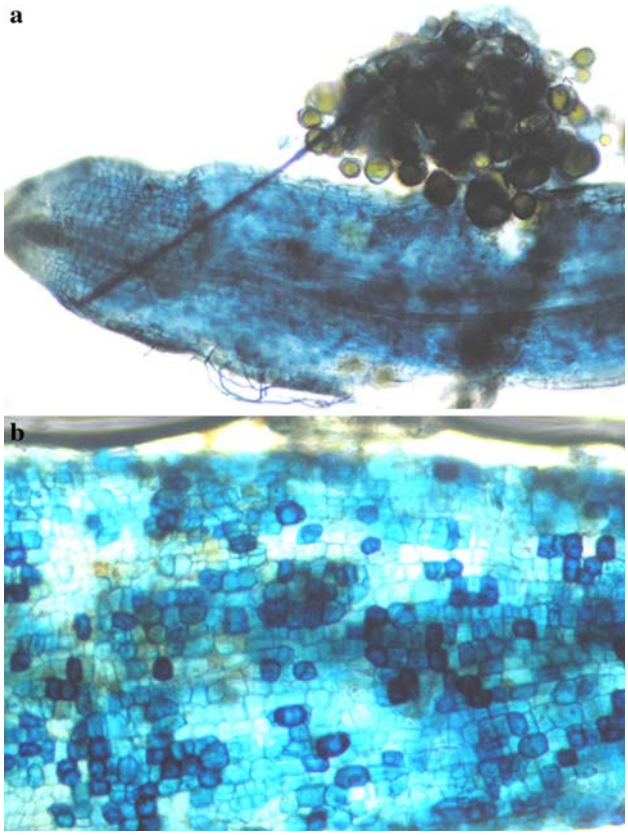

Fig. 1 Extensive vesicle formation in neem roots due to *G. fasciculatum* (*below*) and root tip entangled with extra matricular vesicles (*above*)

the seedling stage, mainly due to higher root colonization, enhanced P uptake and better photosynthetic activity. There have been many reports on AM increasing the root and shoot length, biovolume, dry mass and nutrient uptake in neem seedlings (Muthukumar et al. 2001; Sumana and Bhagyaraja 2006). Seedling growth enhancement and increased mineral uptake were also reported in a number of other annual and perennial plants due to AM (Thakur et al. 2005; Copetta et al. 2006). However, all these studies were on nursery-raised seedlings. Very little is known on the impact of AM on field-grown trees of economic value, in particular on active ingredient quantity in plant parts like roots, leaves, fruits and seeds. In several medicinal and aromatic plants, AM enhanced essential oil content and other bioactive compounds in different parts. Essential oil in leaves of *Ocimum bascillicum* (Copetta et al. 2006), trigonelline (*alkaloid*) in the roots of leguminous tree, *Prosopis laevigata* (Rojas-Andrade et al. 2003) were enhanced due to AM symbiosis. In the present study on the neem tree, we observed a significant impact of AM on oil content, total triterpenoids and azadirachtin in seeds and not on the height and GBH. Since tree growth in the field is a complex process affected by many factors including colonization by native AM, the inoculation affect could not be clearly observed on growth parameters. But the increase in azadirachtin and other triterpenoids could be caused by AM as reported in other plants. Several reports of AM fungi influencing the metabolic pathways in plants have been reviewed recently by Zhi-Lin et al. (2007). Strack and Fester (2006) particularly reported that the terpenoid metabolism in many plants is significantly influenced by mycorrhizal infection. Azadirachtin estimated in the present study belongs to the triterpene group.

One possible mechanism for this improvement may be through enhanced P availability. The experimental site had available P of 9.5 kg/ha, which is classified as low. Further these soils are rich in calcium, which makes P unavailable to the plants, and AM could improve the availability and plant uptake. This seemed possible, as external P application also had similar effect on the azadirachtin content in kernels. Another interesting observation made by us was the seed kernels in the AM-inoculated or P-applied plants remained green even in dried fruits, mainly due to the higher chlorophyll content (0.159 and 0.145 mg/g fresh kernels in AM- and P-applied treatments as against 0.116 mg in control). At harvest, neem seed kernels become brown due to biochemical changes occurring inside, the most important being the development of rancidity and corresponding decrease in the triterpenoid and azadirachtin contents. AM inoculation perhaps helped in slowing down this degradative change. Mycorrhizal association is known to enhance the biosynthesis of cytokinins

Table 2 Growth, yield and azadirachtin content in 5-year-old field-grown neem trees as influenced by AM inoculation and P application

Treatment	Height (m)	GBH (cm)	Fruit yield[a] (kg/tree)	Oil content in kernels (%)	Triterpenoids in kernels (%)	Azadirachtin (a + b) in kernels (%)
Uninoculated	7.1	48	1.7	36.0	2.4	0.45
AM inoculated	7.5	50	2.0	37.5	3.0	0.67
Phosphorus applied	7.3	49	1.7	38.3	2.8	0.61
CD at 5%	NS	NS	NS	0.81	0.2	0.12

[a] Air dried

(Dixon et al. 1988) and enzymes involved in antioxidative metabolism (Porcel et al. 2003) both of which delay degradative changes/senescence.

In conclusion, we observed improved growth and nutrient uptake in neem seedlings inoculated with *G. fasciculatum* in the nursery and higher oil content, triterpenoids and azadirachtin in seed kernels of field-grown plants, though no measurable impact on the height and total fruit yield. This study opens a new management option for producing high azadirachtin from neem trees through AM inoculation.

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