Diversity of endomycorrhizal fungi and their synergistic effect on the growth of *Acacia catechu* Willd.

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ABSTRACT: The diversity of arbuscular mycorrhizal (AM) fungi of *Acacia catechu* Willd. was studied. Dominant AM spores, the bacterium *Rhizobium* sp. along with the fungus *Trichoderma viride* were isolated from the rhizosphere of *A. catechu* and mass-produced in laboratory. The co-inoculation effect of *Glomus mosseae, Glomus fasciculatum*, mixed AM (*Glomus* spp. [except *G. mosseae, G. fasciculatum*] with *Acaulospora* spp., *Sclerocystis* spp. and *Gigaspora* spp.), *Rhizobium* sp. and *Trichoderma viride* was studied as exerted on the growth of *A. catechu* seedlings. All inoculated seedlings showed improved seedling growth compared to the control. Inoculated seedlings had a pronounced effect on all growth parameters such as height, fresh and dry weight of roots and shoots, AM spore count, per cent mycorrhizal colonization in roots and root nodule number in comparison with uninoculated seedlings. Phosphorus uptake was also higher in inoculated seedlings than in the control. This study provides a good scope for commercially utilizing the efficient strains of AM fungi for beneficial effects with other beneficial rhizosphere microflora in the primary establishment of slow growing seedlings ensuring better survival and improved growth.

Keywords: *Acacia catechu* Willd.; co-inoculation; phosphorus; *Rhizobium* sp.; *Trichoderma viride*; AM diversity

Abbreviations: ppm – parts per million, LSD – least standard deviation, sp. – species, AM – arbuscular mycorrhiza

Acacia catechu Willd. is an medicinally important leguminous tree that grows naturally in all kinds of geological formations and soils of all kinds. *Katha*, which is an important ingredient in the "*Pan*" preparation commonly used in northern India, is commercially obtained from this plant. *A. catechu* has better microflora in its rhizosphere. The arbuscular mycorrhizal (AM) fungi take an important ecological position amongst various microorganisms colonizing the rhizosphere of plants. The occurrence of endomycorrhizal fungi (AM) in soil, their association with both forestation and agricultural crops are well documented (Rani et al. 1999; Gill, Singh 2002). Inoculation with a suitable AM fungal strain to improve the growth and survival of plant seedlings in forestation is very essential. The role of AM fungi in improving the quality and survival of plant seedlings after plantation has been well recognized (Kaushik et al. 1992; Khan, Uniyal 1999; Ortas 2003; Boureima et al. 2007). The mutual relationship between mycorrhizal fungi and host plants has been studied in terms of the benefits to individual plants and fungi (Francis, Read 1995; Smith, Smith 1996). The effect of mycorrhizal fungi on nutrient uptake and plant growth has been extensively studied (Theodose, Bowman 1997; Wallander et al. 1999; Liu et al. 2000; Abdel-Hanej, Abdel-Monsif 2006). Mycorrhizal infection has a particular value for legumes because nodulation and symbiotic nitrogen fixation by Rhizobia require an adequate phosphorus supply and a restricted root system leads to poor competition for soil phosphorus (Carling et al. 1978).

Various researchers have made efforts to increase the quality of seedlings of forest trees through the inoculation of a suitable mycorrhizal strain alone

Table 1. Growth response of *Acacia catechu* to double inoculation after 90 days

S. No.	Treatment (double inoculation)	Increase in height (cm)	Root length (cm)	Fresh shoot wt. (g)	Dry shoot wt. (g)	Fresh root wt. (g)	Dry root wt. (g)	VAM spore number/50 g soil	Mycorrhizal colonization in roots (%)	Presence of nodules
1	Control	*1.5 ± 0.03	13.0 ± 0.70	6.8 ± 0.01	5.73 ± 0.02	8.4 ± 0.34	6.62 ± 0.36	39 ± 0.70	56.66 ± 2.72	–
2	Mixed AM + *Rhizobium* sp.	22.0 ± 1.06	21.0 ± 0.70	7.9 ± 0.03	6.37 ± 0.02	10.6 ± 0.69	7.65 ± 0.26	113 ± 2.82	93.33 ± 5.44	++
3	*G. mosseae* + *Rhizobium* sp.	22.2 ± 1.41	22.5 ± 1.76	10.3 ± 0.36	7.89 ± 0.23	14.3 ± 0.40	10.09 ± 5.65	120 ± 2.12	93.33 ± 4.61	+++
4	*G. fasciculatum* + *Rhizobium* sp.	8.25 ± 0.35	20.0 ± 1.41	7.7 ± 0.47	6.20 ± 0.35	8.8 ± 1.08	6.56 ± 0.47	102 ± 0.70	80.55 ± 2.26	+
5	*Trichoderma viride* + *Rhizobium* sp.	4.0 ± 0.35	17.5 ± 5.30	7.2 ± 0.42	6.01 ± 0.15	8.5 ± 0.61	6.81 ± 0.13	101 ± 1.41	76.66 ± 2.72	–

*Mean of five replications each, ± standard error, +++ abundant, ++ moderate, + low, – absent

Source of variation	Degree of freedom	Sum of squares	Mean of squares	*F* obtained (*F* ratio)	*F* crit. (table)
Between	4	441	110.25	15.7*	0.05 $P = 5.19$
Within	5	32	6.4		0.01 $P = 11.3$
Total	9	473			

*According to the analysis of variance (ANOVA) of data, the calculated F ratio is 15.7, which is significant at 0.01% P level (F crit. 0.01 = 5.19, 0.05 = 11.39) or AM-*Rhizobium* sp. combinations in nursery conditions (Azcon, Rubio 1990; Bethlenfalvay et al. 1990; Champawat 1990; Reena, Bagyaraj 1990; Leopold, Hofner 1991; Thiagarajan et al. 1992; Saxena, Tilk 1997; Rani et al. 1999; Kumar et al. 2002; Parkash et al. 2005; Khan, Zaidi 2007). But very little or hardly any work has been done to improve and produce the quality seedlings of *A. catechu* by using the triple co-inoculation of VAM strains since this plant is economically more important on a local level and slow growing as well. Bearing this in mind, the present study was undertaken to determine the effect of double and triple inoculation (co-inoculation) of AM fungi with other rhizospheric microflora on the growth performance of *A. catechu*.

MATERIALS AND METHODS

The native predominant AM fungi, i.e. *Glomus mosseae* and *Glomus fasciculatum*, were isolated from the rhizosphere of *A. catechu*. All the remaining species of *Glomus* (except *G. mosseae, G. fasciculatum*), *Acaulospora*, *Sclerocystis* and *Gigaspora* were also isolated and mixed together to prepare a mixed AM inoculum. *Rhizobium* sp. and *Trichoderma viride* were also isolated from the rhizosphere of *A. catechu* in a similar way. All mycorrhizal inocula (*Glomus mosseae, Glomus fasciculatum,* Mixed AM) were mass-produced on maize (*Zea mays* L.). *Rhizobium* sp. inoculum was mass-cultured on yeast extract mannitol agar (YEMA) medium. *T. viride* was also mass-produced on wheat bran:saw dust:water (3:1:4) medium for further inoculation experiments. Seedlings of *A. catechu* were procured from Divisional Forest Nursery, Hamirpur Forest Department, Himachal Pradesh, India.

Inoculation*:* Inoculation experiments were designed in double and triple combinations (co-inoculation). In double inoculation, mixed AM (except *G. mosseae* and *G. fasciculatum*), *G. mosseae, G. fasciculatum* and *Trichoderma viride* were mixed with *Rhizobium* species only while in triple inoculation, mixed AM (except *G. mosseae* and *G. fasciculatum*), *G. mosseae* and *G. fasciculatum* were mixed together with *T. viride* and *Rhizobium* species. All inoculation experiments were performed in sterilized soils. Two seedlings of *A. catechu* were grown in each experimental earthen pot (30 × 30 cm) in a sandy soil mixture (300:1,500 g). To each pot 10% of the fungal (*T. viride*), bacterial (*Rhizobium* sp.) and mycorrhizal inocula along with infected roots were added around the rhizosphere. Only five replications were prepared. In control sets, no inoculum was added. After 45 and 90 days interval observations were recorded on seedling shoot length (increase in height), root length, shoot and root fresh and dry weight, percentage mycorrhizal colonization in roots, AM spore number, root nodule number and phosphorus content (%) of seedlings. But only the observations after 90 days are cited in this paper. Percentage mycorrhizal colonization in roots was studied (Phillips, Hayman 1970). The AM spore quantification was determined (Gerdemann, Nicolson 1963). Phosphorus content (%) of shoots and roots was determined by vanadomolybdate phosphoric yellow colour method (Jackson 1973). Data were compared with the control after treatment. The data were analyzed statistically by the analysis of variance (ANOVA) (Pagano 2000).

RESULTS AND DISCUSSION

Four genera of VAM fungi, e.g. *Glomus, Acaulospora, Gigaspora* and *Sclerocystis*, have been reported from the rhizosphere of *A. catechu*. *Entrophospora* and *Scutellospora* were found absent in the rhizosphere. *Glomus* spp. were present in abundance compared to the other genera. The percentage mycorrhizal colonization in roots was (90 ± 7.07) and VAM spore number was (182.5 ± 5.30) in 50 g of soil (five random replications). Both arbuscular and vesicular types of colonization were found in roots of *A. catechu*. The AM fungi present in the rhizosphere of *A. catechu* were *Glomus mosseae, G. fasciculatum, G. intraradices, G. macrocarpum, Acaulospora laevis, A. foveata, Gigaspora* sp., *Sclerocystis coremioides*.

Double inoculation: The effect of double inoculation after 90 days on *Acacia catechu* is shown in Table 1. It was revealed that the *G. mosseae* + *Rhizobium* sp. combination showed a maximum significant (F_{ratio} 15.7, $P > 0.05$) increase in height (22.2 ± 1.41) compared to the other treatments. AM spore numbers, percentage mycorrhizal colonization in roots, increase in plant height, fresh and dry weights (shoot and root) were increased with the time period when compared with earlier data of 45 days. The presence of nodules was also high in *G. mosseae* and *Rhizobium* sp. treatment (Table 1). No nodule was present either in the control or in *Trichoderma viride* + *Rhizobium* sp. treatments.

Triple inoculation: The response of *A. catechu* to triple inoculation after 90 days is shown in Table 2. It is evident from the table that the *G. mosseae* + *Rhizobium* sp. + *Trichoderma viride* (triple inoculum) combination showed a maximum significant (F_{ratio} 48.0, $P > 0.05$) increase in height, root length,

Table 2. Growth response of *Acacia catechu* to triple inoculation after 90 days

S. No.	Treatment (triple inoculation)	Increase in height (cm)	Root length (cm)	Fresh shoot wt. (g)	Dry shoot wt. (g)	Fresh root wt. (g)	Dry root wt. (g)	VAM spore number/50 g soil	Mycorrhizal colonization in roots (%)	Presence of nodules
1	Control	*1.5 ± 0.03	13.0 ± 0.70	6.8 ± 0.01	5.73 ± 0.02	8.4 ± 0.34	6.62 ± 0.36	39 ± 0.70	56.66 ± 2.72	–
2	Mixed AM + *Rhizobium* sp.+ *Trichoderma viride*	11.2 ± 0.70	24.0 ± 5.65	9.9 ± 1.14	7.61 ± 0.74	13.7 ± 2.03	9.65 ± 0.95	118 ± 3.53	80.0 ± 4.71	++
3	*G. mosseae* + *Rhizobium* sp. + *Trichoderma viride*	22.2 ± 0.03	31.0 ± 6.36	10.6 ± 0.95	7.85 ± 0.53	20.5 ± 3.39	13.08 ± 2.09	126.5 ± 4.59	100.0 ± 0	+++
4	*G. fasciculatum* + *Rhizobium* sp. + *Trichoderma viride*	5.88 ± 1.41	19.0 ± 0.70	7.8 ± 0.35	6.37 ± 0.20	10.4 ± 5.65	7.82 ± 0.01	95 ± 3.53	83.33 ± 2.72	+

*Mean of five replications each, ± standard error, ++ moderate, +++ abundant, + low, – absent

Source of variation	Degree of freedom	Sum of squares	Mean of squares	*F* obtained (*F* ratio)	*F* crit. (table)
Between	3	288	96	48.0*	0.05 $P = 3.11$
Within	4	8	2		0.01 $P = 5.06$
Total	7	295			

*According to the analysis of variance (ANOVA) of data, the calculated *F* ratio is 48.0, which is significant at 0.01% *P* level (*F* crit. 0.01 = 6.59, 0.05 = 16.69) fresh and dry weights of shoot and root, AM spore number and percentage mycorrhizal colonization in roots after 90 days in comparison with the other inoculated treatments and control in which all these above-mentioned growth parameters were low. In this case, the *G. mosseae* + *Rhizobium* sp. + *Trichoderma viride* treatment also had more nodules than the other treatments. Nodules were absent in the control.

Double inoculation of mixed AM + *Rhizobium* sp. also showed a higher P content (%) in shoots (0.48 ± 0.01) and roots (0.99 ± 0.01) than the other treatments as well as the control (Table 3) whereas the triple inoculation of mixed VAM + *Rhizobium* sp. + *Trichoderma viride* showed a higher P content of roots (0.99 ± 0.01) than all the other treatments and control. P content of shoots (0.48 ± 0.01) was higher in all treatments than in the control and it was also equal to the *G. mosseae* + *Rhizobium* sp. + *Trichoderma viride* treatment (0.48 ± 0.01). It is evident from the results that all the inoculations had a higher P content of shoots and roots than the control. P content of roots was higher than P content of shoots in all the inoculated seedlings of *A. catechu*.

There are many reports that suggest the importance of VAM fungi in producing quality seedlings. Recently, papers were published in which the positive growth effect and nutrient uptake by plants through AM inoculation was seen under different water regimes and bioremediated and agricultural soils (Abdel-Hanej, Abdel-Monsief 2006; Al Zalzaleh et al. 2009). Jinying et al. (2007) studied the effect of AM inoculation on the drought resistance of wild jujube (*Ziziphus spinosus* Hu) seedlings. They found that arbuscular mycorrhizal fungi helped withstand the water stress and also improved the growth of the seedlings.

In the present study, a soil-based inoculum was used for all the experiments. Hence, the better growth responses were seen in *A. catechu*. This might be due to higher reproduction of AM fungi present in the soil-based inoculum, which sprouted rapidly from extracellular and intracellular hyphae present in the soil and root inoculum. Co-inoculation has a synergistic effect on seedlings through increasing the efficiency of the shoot and root system in providing the plant with essential levels of P and N for growth. Inoculated seedlings with the entire test AM fungi increased the phosphorus content of roots and shoots as compared to the control in this study. The increased rate of phosphorus uptake and inflow in roots has been regarded as the major contribution of AM infection (Mosse 1973). Present findings also indicated that mycorrhizal co-inoculated seedlings

Table 3. Effect of co-inoculation on the phosphorus content of *Acacia catechu*

S. No.	Treatment	Phosphorus content (%) (ppm)	
		*shoot	*root
Double inoculation			
1	Mixed AM + *Rhizobium* sp.	$0.48^{a} \pm 0.01$	$0.99^{d} \pm 0.01$
2	*G. mosseae* + *Rhizobium* sp.	$0.46^{a} \pm 0.02$	$0.91^{d} \pm 0$
3	*G. fasciculatum* + *Rhizobium* sp.	$0.44^{a} \pm 0$	$0.79^{c} \pm 0.01$
4	*Trichoderma viride* + *Rhizobium* sp.	$0.43^{a} \pm 0.02$	$0.61^{b} \pm 0.01$
5	Control	$0.41^{a} \pm 0$	$0.43^{a} \pm 0$
	LSD	0.37	0.12
Triple inoculation			
1	Mixed AM + *Rhizobium* sp. + *Trichoderma viride*	$0.48^{a} \pm 0.01$	$0.99^{b} \pm 0.01$
2	*G. mosseae* + *Rhizobium* sp. + *Trichoderma viride*	$0.48^{a} \pm 0$	$0.89^{b} \pm 0.021$
3	*G. fasciculatum* + *Rhizobium* sp. + *Trichoderma viride*	$0.46^{a} \pm 0.023$	$0.88^{b} \pm 0.010$
4	Control	$0.41^{a} \pm 0$	$0.43^{a} \pm 0$
	LSD	0.25	0.13

*Average of five replications each, means followed by the same superscript letter in columns are not significant at 0.05 *P* level, ± standard error of mean had a higher phosphorus content than the control and similar results were also reported (Kumar et al. 2002) on chickpea. Zhao-Yuhua et al. (1997) observed that nodulation and plant growth were affected by the degree of mycorrhization, i.e. both were increased at higher levels. Interactions between the host and symbiont also varied with cultivars. Kumar et al. (2002) concluded that mycorrhiza or *Azotobacter* or *Rhizobium* alone or their combinations could have an important effect on nodulation and nitrogen fixation in legumes. The principal effect of mycorrhiza on nodulation is P-mediated. The combined inoculations of symbionts showed significantly increased N-fixations growth and nutrient uptake in *Leucaena leucocephala* and *Cajanus cajan* (Sekhon et al. 1992).

The mutual association accounted for better colonization and plant growth due to the interchange of carbon, phosphate and nitrogen between the host fungi and bacteria. According to Bader-El-Din and Moawad (1998), the dual inoculation of AM and *Rhizobium* had a synergistic effect on nodulation, plant growth, dry matter production and nitrogen fixation. Increased N-fixation due to increased mycorrhizal colonization in roots and nodulation may contribute to the growth and yield of plants (Gill, Singh 2002). Jamaluddin et al. (2001) reported that the growth of plants was enhanced by mycorrhizal infection by increasing nutrient uptake via an increase in the absorbing surface area of roots. Similar results were also obtained on wheat with plant growth promoting rhizobacteria 'PGPR' (Khan, Zaidi 2007). Dodd and Thompson (1994) also observed similar results while using the soil-based AM inoculum. They reported that the spore population was maximum in soil root based inoculum followed by AM spores inoculum. Recognition of the complexity of interactions among microbes in the rhizosphere has led to dual or co-inoculation of crops with both AM fungi and other rhizosphere microorganisms. By virtue of their interdependent and synergistic effects on P and N uptake, interactions between AM fungi and symbiotic N_2-fixing bacteria are important when considering co-inoculations (Ganry et al. 1985; Rao et al. 1986). Nodulation has been shown to be sometimes dependent on colonization by VAM fungi (Azcon-Aguilar, Barea 1978). Similarly, inoculations with selected plant growth promoting rhizosphere bacteria have demonstrated synergistic benefits to the host when co-inoculated with VAM fungi. In many cases, colonization in the roots by AM fungi was increased by the other inoculants (Azcon-Aguilar, Barea 1978; Ames et al. 1989). It has also been reported in this study that the effect of AM fungi is increased when they were co-inoculated with other rhizosphere microflora like *Rhizobium* sp. and *Trichoderma viride*.

CONCLUSION

All double and triple inoculation (co-inoculation) treatments have a significant marked effect on the growth of *A. catechu* seedlings compared to the control sets in which a very small growth effect was observed. In double inoculation, the *G. mosseae* + *Rhizobium* sp. combination had a higher growth effect than the other treatments. In triple inoculation, *G. mosseae* + *Rhizobium* sp. + *Trichoderma viride* had a higher growth effect than the other treatments on *A. catechu* seedlings. This is due to the mutual positive action of *Rhizobium* sp. and AM fungi strains that helped in uptake of P and N from soil. On the other hand, *Trichoderma viride* being a biocontrol agent helped to control all pathogenic fungal attack in the rhizosphere of *A. catechu*. On the basis of the results it can be concluded that the co-inoculation of *Rhizobium* sp. and *Trichoderma viride* with efficient strains of AM fungi should be used in practice to produce improved seedlings of *A. catechu* ensuring better survival and improved growth during adverse conditions in outplanted seedlings.

Acknowledgements

Financial support from the Council of Scientific and Industrial Research, New Delhi, India, rendered to Vipin Parkash is duly acknowledged.

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Received for publication December 24, 2008
Accepted after corrections April 28, 2009

Diverzita endomykorhizních hub a jejich synergistický efekt na růst *Acacia catechu* Willd.

ABSTRAKT: Byla studována diverzita vesikulo-arbuskulárních mykorhizních (VAM) hub stromu *Acacia catechu* Willd. Spóry dominantních druhů VAM hub, bakterií *Rhizobium* sp. a houby *Trichoderma viride* byly izolovány z rizosféry stromu *A. catechu* a namnoženy v laboratoři. Hlavním cílem práce bylo studium vlivu vícenásobné inokulace VAM druhů *Glomus mosseae a Glomus fasciculatum*, směsi VAM druhů (*Glomus* spp. [kromě *G. mosseae, G. fasciculatum*] s *Acaulospora* spp., *Sclerocystis* spp. a *Gigaspora* spp.), bakterií *Rhizobium* sp. a houby *Trichoderma viride* na růst semenáčků dřeviny *A. catechu*. Všechny očkované semenáčky vykázaly zlepšený růst proti kontrole, a to ve všech sledovaných parametrech (výška, čerstvá hmotnost a sušina kořenů a prýtů, počet spór VAM hub, procento mykorhizní kolonizace kořenů a počet hlízek bakterií *Rhizobium*). Příjem fosforu byl také proti kontrole vyšší. Výsledky prokázaly možnosti komerčního využití efektivních kmenů VAM hub společně s dalšími rizosferními mikroorganismy pro zajištění lepšího vzcházení a dalšího růstu semenáčků dřeviny *A. catechu*.

Klíčová slova: *Acacia catechu* Willd.; vícenásobná inokulace; fosfor; *Rhizobium* sp.; *Trichoderma viride*; VAM diverzita

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