

Effect of AM fungal association with normal and micropropagated plants of *Andrographis paniculata* Nees on biomass, primary and secondary metabolites

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Andrographis paniculata Nees commonly known as “King of bitters” is one of the promising herbs used in the treatment of several ailments including human cancers and the myriad of symptoms associated with auto immune disorders. The main active principle which is therapeutically known is Andrographolide. The beneficial effects of arbuscular mycorrhizal (AM) fungal association with medicinal plants are well established. Both normal and micropropagated plants of *A. paniculata* were treated with *Glomus mosseae* and *G. fasciculatum* to study the effects of AM association on morphology and physiology of growth. General growth performance, chlorophyll, primary and secondary metabolite contents in both control and treated normal and micropropagated plants were estimated with standard procedures and comparisons were made. The percent of colonization and the number of spores in the rhizosphere of the micropropagated plants are significantly more than the normal plants. The contents of chlorophylls and the primary metabolites had enhanced in the micropropagated plants in response to the mycorrhizal association. HPLC analysis of andrographolide revealed that *Glomus fasciculatum* treated micropropagated plants could synthesis higher percent of the active principle followed by normal plants inoculated with the same species of *Glomus*. It was found that micropropagated plants inoculated with *G. fasciculatum* performed better than all other samples in its growth performance and contents of primary and secondary metabolites.

Keywords: *Andrographis paniculata*, micropropagated plants, AMF, growth performance, andrographolide, HPLC.

INTRODUCTION

Andrographis paniculata Nees is one of the extensively used medicinal plants in India for several herbal preparations with export marketing potentials (Anandalwar and Vasantha, 1989). The whole plant contains an appreciable amount of Andrographolide, which is known as a hepatoprotective. The plant also contains andrographolide esters and lactones that have been found to be cancerolytic and antiviral, apart from wide range of other pharmacological uses. In India alone,

the pharmaceutical companies require nearly 3000 tones of plants per annum exclusively for manufacturing different products (Anandalwar and Vasantha, 1989).

Andrographis paniculata is mainly propagated through seeds and exhibits a significant phenotypic heterogeneity among the populations growing at different locations (Sabu et al., 2001). Since the viability of seeds is not very significant the *in vitro*, micropropagation technology is being used effectively for meeting the demands of genetically uniform planting stock with vigorous growth. The *in vitro* culture techniques have also gained importance as methods for enhancing the production of secondary metabolites (Vanisree et al., 2004) and there are many reports on the enhancement of secondary

metabolism in micropropagated plants than the source of explants (Giulietti and Ertola, 1999; Tejavathi and Rao, 1998; Leal et al., 2009).

Though the micropropagation is an established technique for the production of elite clones, the plants undergo a transient transplantation shock and require biological hardening after transplantation. Inoculation of compatible arbuscular mycorrhiza (AM) fungi during the acclimatization period of micropropagated plants during hardening has emerged as an alternative strategy for better establishment by improving the plant growth (Rai 2001) and the acclimatization period can also be shortened (Salamanca et al., 1992).

The beneficial effects of AM fungal association on the growth performances of several taxa including medicinal plants are well documented (Gianinazzi and Gianinazzi-Pearson, 1988). Reviews concerning micropropagation and mycorrhization have also been published by Lovato et al., (1996); Rai (2001) and Kapoor et al., (2008). AM fungal association enhances the plant growth as a result of improved nutrient uptake and consequently leading to better growth performance of plants. In addition, AM fungi also have an influence on the primary and secondary metabolism in the host (Maier et al., 1997; Peipp et al., 1997). There are a few reports on the potential effects on the enhancement of metabolites with active principles in medicinal plants (Kapoor et al., 2002a, b; Copetta et al., 2006; Arpana and Bagyaraj, 2007; Karthikeyan et al., 2008, Tsai and Phillips, 1991). The beneficial AM fungal association with micropropagated plants prompted us to study the effect of selected AM fungi on biomass, primary and secondary metabolites in normal as well as micropropagated plants of *A. paniculata*.

MATERIALS AND METHODS

Plant Source

Plants raised from seeds (hereafter referred to as normal plants) and micropropagated plants were inoculated with AM fungi. Normal plants were raised from the healthy seeds procured from University of Agricultural Sciences, GKVK, Bangalore and the micropropagated plants were obtained through cotyledonary nodal cultures. Multiple shoot regeneration was obtained on a MS medium containing BAP (8.86 μ M). Thus obtained shoots were subcultured to auxin supplemented medium for rooting. The rooted shoots were sequentially hardened by transferring them to sterile distilled water to vermiculate and finally soil (Tejavathi et al., 2008).

Experimental design

Pot culture experiments were conducted with two AM fungi, *Glomus mosseae* (T.H. Nicolson and Ged.) Gerd.

and Trappe BEG 12 and *G. fasciculatum* (Thaxt.) Gerd. and Trappe emend.C.Walker and Koske, procured from the Department of Microbiology, University of Agricultural Sciences, GKVK, Bangalore and maintained in pot cultures with 5 Kg of sterilized sand: soil (1:1) mixture as substrate on Rhodes grass (*Chloris gayoma*). The experimental pots were filled with sterilized soil and sand mixture (5Kg) in the ratio of 1:1 (v/v) and the AM fungal inoculum consisted of sand and soil mixture containing spores, extramatrical mycelia and mycorrhizal root segments of Rhodes grass were added at the rate of 25 g per pot (28 x 24 x 12: upper diameter, basal diameter and height in cm) with 106 infective propagules per gram of substrate. The pots filled with sand and soil without the inoculum was maintained as control. Seedlings of both normal and micropropagated plants of one month old were transplanted to both treated and control pots and maintained in a poly house. Experiment was designed in randomized block consisted of three different treatments-nonmycorrhizal, inoculated with *Glomus mosseae* or with *G. fasciculatum* in 5 replicates.

Experiments included six treatments namely

1. Normal plants without AM fungi
2. Normal plants inoculated with *Glomus mosseae*
3. Normal plants inoculated with *G. fasciculatum*
4. Micropropagated plants without AM fungi
5. Micropropagated plants inoculated with *G. mosseae*
6. Micropropagated plants inoculated with *G. fasciculatum*

Determination of root colonization

The percent AM fungal colonization in normal and micropropagated plants was estimated (Phillips and Hayman, 1970) at an interval of 2, 3 and 4 months of inoculation since the life span of *A. paniculata* is about 5 to 6 months. Assessment of roots for AM fungal colonization was made on random sampling of the root system. Root samples were cut into 1 cm segments, and cleared by autoclaving with 10% KOH at 108 kpi for 15 min. The alkalinity was neutralized with 10% HCl and staining was done with 0.03% trypan blue in lactoglycerol. The stained preparation was scanned under microscope for the presence of AM fungal structures and the percent colonization was calculated using the formula

$$\% \text{ Colonization} = \frac{\text{No. of root bits with AM fungal structures}}{\text{Total No. of root bits examined}} \times 100$$

Enumeration of AM fungal spores

The extrametrical chlamydospores produced by the

mycorrhizal fungus were estimated by a wet sieving and decanting method (Gerdemann and Nicolson, 1963). One hundred grams of representative soil samples from each treatment was suspended in known quantity of water and stirred thoroughly. Resulting soil suspension was passed through nested sieves of 1000, 300, 205, 105, 45 μm in the same order. The soil and the spores on the bottom two sieves were transferred onto a nylon mesh of the same size as the last sieve. The nylon mesh with spores on it was placed on a Petri dish and the spores were counted under the microscope.

Morphological evaluation

The morphological and yield characters were studied in control and treated normal and micropropagated plants periodically at 2, 3 and 4 months after inoculation. Morphological characters like root and shoot length, number of branches and leaf parameters were recorded. Leaf surface area was measured using imageJ software (<http://rsb.info.nih.gov/ij/>). Yield parameters including biomass, number of flowers fruits and seeds were recorded after 4 months of treatment. For each parameter 12 plants were randomly selected and data was subjected to statistical analysis (Table 2 and 3). Stomatal index was calculated by the following formula

$$\text{Stomatal index} = \frac{\text{No. of stomata per unit leaf area}}{\text{No. of stomata per unit leaf area} \times \text{No. of epidermal cells per unit leaf area}} \times 100$$

Estimation of chlorophyll content

Chlorophyll a, b and total chlorophyll content in fresh leaves of six samples at the intervals of 2,3 and 4 month were estimated (Arnon, 1949).

Estimation of primary metabolites

Total carbohydrates and reducing sugars contents in root stem and leaves of six samples collected at an intervals of 2,3 and 4 months after inoculation were estimated following anthrone and DNS reagent methods respectively (Yemm and Willis, 1954; Miller, 1959). Total proteins and amino acids were analyzed following Lowry et al., (1951) and Moore and Stein (1954) methods respectively.

Estimation of secondary metabolites

Total phenolics in root, stem and leaf segments of six samples were estimated following Folin-Ciocalteu rea-

gent method of Slinkard and Singleton (1977) using gallic acid as a standard phenolic compound at an interval of 2, 3 and 4 months of inoculation.

HPLC analysis of Andrographolide

Andrographolide content in all the samples were quantified by high performance liquid chromatography (HPLC). Leaves of both control and treated samples at 120 days old of AM inoculation were used for HPLC analysis. 2.5 g of coarsely powdered samples were extracted with 50 ml of HPLC grade methanol. The extract was refluxed on a water bath for 15 min and filtered. The residue was further refluxed with methanol till the last extract turns colorless, and filtered. The filtrates were concentrated to 50 ml. Concentration of reference sample andrographolide was 0.01 % W/V in methanol. Shimadzu Lc-Z010a liquid chromatograph was used with a RP8 lichrosphere, 5 μ Lichrocart 250-4 mm column. The mobile phase was methanol and water (65:35) with a flow rate of 1.5 ml/min. All the six samples were filtered through 0.22 μm filter and sonicated to remove any gas, 20 μl of samples were injected and the chromatograms were recorded at 223 nm (Figure 1).

Data analysis

The data was subjected to statistical analysis - two way ANOVA, significant 'F' ratios between group means were further subjected to Fisher's Protected LSD test using SPSS version 15. Probability (P) values <0.05 were considered significant.

RESULTS

AM Fungal colonization

The percent of colonization, number of vesicles and arbuscules and sporulation in the rhizosphere increased with time. The micropropagated plants inoculated with *G. fasciculatum* recorded 40% colonization, whereas the normal plants inoculated with the same species showed 35% of colonization. Both the AM fungi have colonized better the micropropagated plants than the normal plants. The sporulation was also found to be more in the rhizosphere of micropropagated plants inoculated with *G. fasciculatum* and showed positive correlation with the number of vesicles and arbuscules (Table 1).

Morphological evaluation

Growth performance of the micropropagated plants inoculated with *G. fasciculatum* in general was better

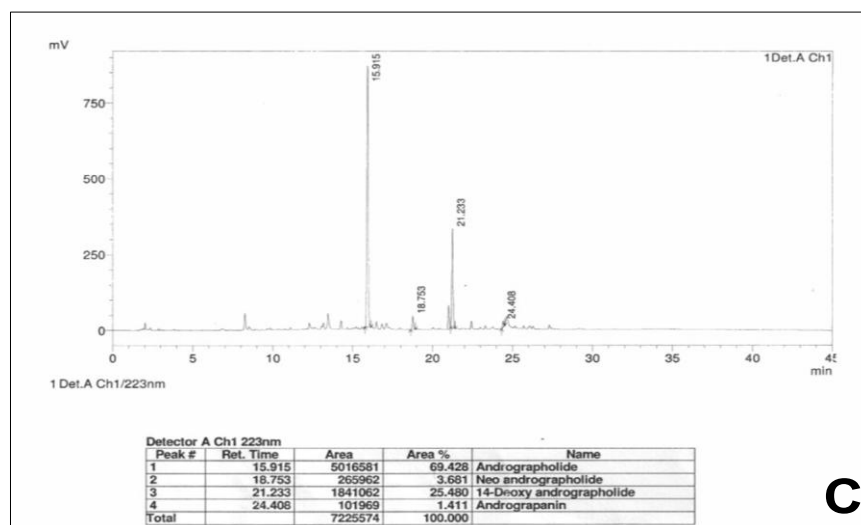
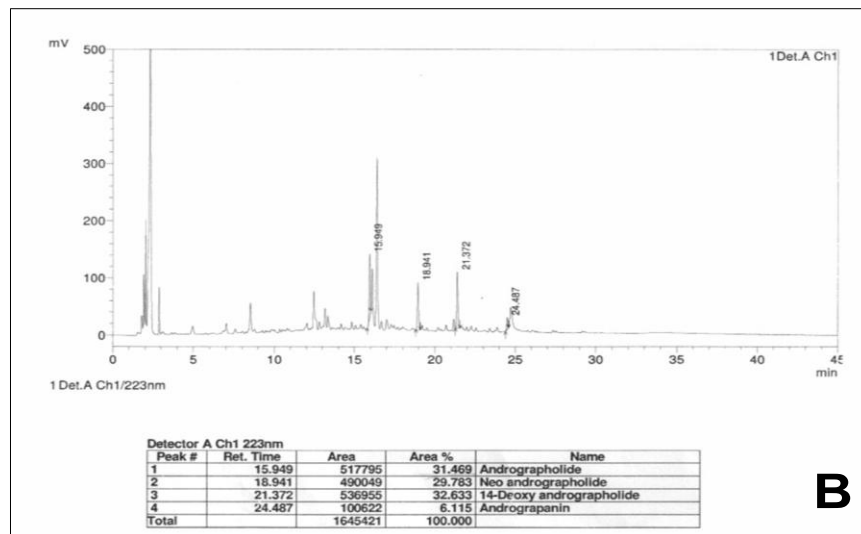
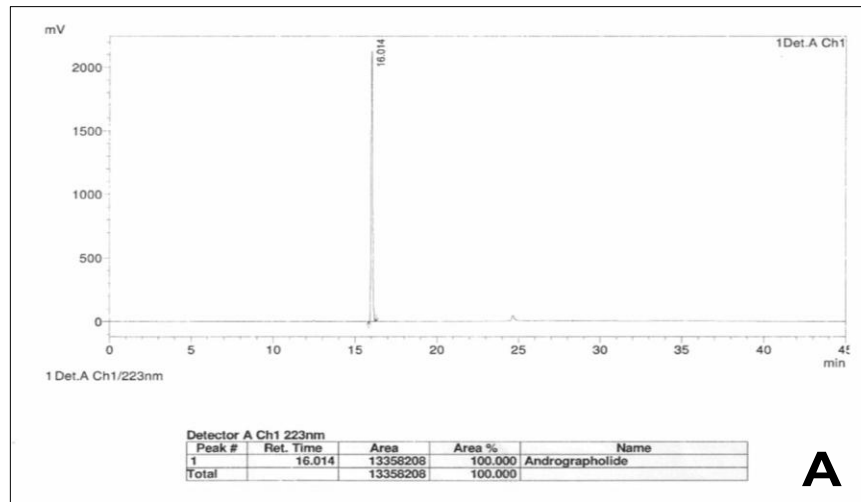


Figure 1. HPLC chromatograms. A: Standard Andrographolide, B: Andrographolide content in Normal plant treated with *Glomus fasciculatum*, C: Andrographolide content in micropropagated plant treated with *Glomus fasciculatum*.

Table 1. Percent of colonization, number of vesicles, arbuscles and spore count in normal and micropropagated plants of *A. paniculata* treated with *Glomus mosseae* and *Glomus fasciculatum* after 4 months of treatment

Treatments	Percentage of colonization	Number of vesicles(per cm root)	Number of arbuscles(per cm root)	Spore count(per gram soil)
Normal <i>G. mosseae</i>	28.00 ±1.63*	33.00 ±0.39*	35.00 ±0.58*	78.00 ±0.54*
Normal <i>G. fasciculatum</i>	35.00 ±0.88*	42.00 ±0.74*	39.00 ±0.66*	100.00 ±0.54**
Micropropagated <i>G. mosseae</i>	32.00 ±0.72*	40.00 ±0.89*	39.00 ±0.50*	95.00 ±0.54**
Micropropagated <i>G. fasciculatum</i>	40.00 ±0.70*	75.00 ±0.20**	76.00 ±0.48**	130.00 ±0.44**

* The mean differences are significant at P < 0.05 as determined by Fischer's protected LSD test

** The mean differences are highly significant at P < 0.01 as determined by Fischer's protected LSD test

compared to other treatments (Tables 2 and 3) after four months of inoculation (Table 2). Highest biomass of 33.20 g was recorded in 5 months old micropropagated plants inoculated with *G. fasciculatum* and the lowest biomass of 16.5 g was recorded in normal control plants of the same age (Table 3). The number of days taken for flowering showed variations among normal and micropropagated plants. It is less in normal control compared to normal AM inoculated plants. Further, it is less in the *G. fasciculatum* inoculated micropropagated plants (60 days) than the micropropagated control (80 days) and normal control (65 days) plants. The number of fruits per plant also shows variation among the normal and micropropagated plants. It is more in normal plants inoculated with *G. fasciculatum* (14 no.) compared to control normal plants (10 no.). Whereas the micropropagated control plant yielded 20 fruits on average per plant, the AM inoculated micropropagated plants could bear only 7 in *G. mosseae* inoculated and 8 in *G. fasciculatum* inoculated plants (Table 2). Data recorded in both control and treated normal and micropropagated plants revealed that vegetative growth was more pronounced in the control and treated micropropagated plants than the control and treated normal plants. The surface area of the leaves in micropropagated plant inoculated with *G. fasciculatum* was found to be 53 cm² as against 39 cm² in normal plant. Stomatal index values were also varied among the treatments and high in micropropagated plants inoculated with *G. fasciculatum* compared to other treatments (Table 3).

Chlorophyll content

Chlorophyll-a, chlorophyll-b and total chlorophyll contents were significantly more in both control and mycorrhiza micropropagated plants. Among the treatments, inoculation with *G. fasciculatum* enhanced the synthesis

of these pigments both in normal and micropropagated plants (Table 4).

Primary metabolites contents

Though the protein and amino acid content in inoculated plants enhanced significantly, carbohydrates decreases in the treatments (Table 5). However, the contents of reducing sugars showed variation among the normal and micropropagated plants. In normal plants, the leaf, stem and root of control plants contain more quantities of reduced sugars than the AM inoculated plants. While in micropropagated plants inoculated with *G. fasciculatum*, root and leaf contain more reducing sugars than the control. As shown in the Table 5 micropropagated plants inoculated with *G. fasciculatum* contained more reducing sugars in leaves and roots, whereas proteins and amino acids were more in leaf, stem and roots.

Secondary metabolite content

Total phenolics were significantly higher in micropropagated plants compared to normal plants especially in *G. fasciculatum* treated micropropagated plants. Total phenolics recorded in the roots of *G. fasciculatum* inoculated micropropagated plants was found to be higher than in the stem and leaves.. Even in normal plant inoculated with *G. fasciculatum* root contained 430.7 µm/g of total phenolics that was more than in the stem and leaf samples (Table 6). HPLC analysis of all the six samples revealed that micropropagated plants inoculated with *G. fasciculatum* contained highest percent of andrographolide (1.05%) followed by normal plant treated with the same AM species (0.73%) (Table 6). Micropropagated control plants had slightly more quantity (0.72%) of active principles than normal control plants (0.61%) which was

Table 2. Influence of AM fungal association on growth performance after 4 months of treatment

Treatments	Plant height (cm)	Number of nodes	Number of branches		Internodal length (cm)	Number of flowers	Number of fruits	Number of seeds
			Main	Lateral				
Normal	42.00 ± 0.24	50.00 ±0.29	2.00 ±0.20	10.00 ±0.66	7.00 ±0.37	15.00 ±0.37	10.00 ±0.73	8.00 ±0.37
Normal <i>G. mosseae</i>	46.00 ± 0.31*	67.00 ±0.86*	2.00 ±0.20	11.00 ±0.20	7.00 ±0.54	14.00 ±0.37	9.00 ±0.70	9.00 ±1.20
Normal <i>G. fasciculatum</i>	60.00 ± 1.05*	75.00 ±0.86*	2.00 ±0.20	13.00 ±0.37*	8.00 ±0.44	21.00 ±0.70*	14.00 ±0.70*	9.00 ±0.80
Micropropagated	52.00 ± 0.40*	68.00 ±0.41*	3.00 ±0.24	15.00 ±0.40*	9.00 ±0.73	24.00 ±0.87*	20.00 ±0.58*	9.00 ±0.44
Micropropagated <i>G. mosseae</i>	59.00 ± 0.58*	78.00 ±1.30*	2.00 ±0.20	17.00 ±0.80*	9.00 ±0.73	12.00 ±0.37	7.00 ±0.31	8.00 ±0.15
Micropropagated <i>G. fasciculatum</i>	64.00 ± 0.64*	80.00 ±0.50*	3.00 ±0.24	22.00 ±0.24*	9.00 ±0.74	13.00 ±1.80	8.00 ±0.66	9.00 ±0.45

* The mean differences are significant at P < 0.05 as determined by Fischer’s protected LSD test

Table 3. Effect of AM fungal association on biomass, stomatal index and growth parameters after 4 months of treatment

Treatments	Biomass (g)		Root length (cm)	Shoot length (cm)	Leaf length (cm)	Leaf width (cm)	Leaf surface area (cm ²)	Stomatal index
	Fresh weight	Dry weight						
Normal	16.50 ±0.20	6.85 ±0.24	12.00 ±0.31	44.00 ±0.54	6.20 ±0.24	3.00 ±0.24	39.00 ±0.48	17.80 ±0.37
Normal <i>G. mosseae</i>	19.00 ±0.37*	8.05 ±0.24	18.00 ±0.24*	46.00 ±0.48*	6.50 ±0.24	2.20 ±0.24	40.00 ±0.58	21.90 ±0.44
Normal <i>G. fasciculatum</i>	30.40 ±0.48*	12.34 ±0.24*	15.00 ±0.24*	50.00 ±0.96*	6.50 ±0.24	2.00 ±0.22	39.00 ±0.48	22.20 ±0.24
Micropropagated	21.00 ±0.24*	8.90 ±0.20	22.00 ±0.24*	54.00 ±0.58*	7.00 ±0.24	2.50 ±0.24	43.00 ±0.37*	20.00 ±0.20
Micropropagated <i>G. mosseae</i>	23.20 ±0.24*	9.85 ±0.31	24.00 ±0.20*	56.00 ±0.44*	7.50 ±0.20	3.00 ±0.23	46.00 ±0.37*	25.00 ±0.31*
Micropropagated <i>G. fasciculatum</i>	33.20 ±0.48*	13.50 ±0.24*	28.00 ±0.37*	60.00 ±0.37*	8.00 ±0.24	3.00 ±0.24	53.00 ±0.48**	29.40 ±0.87*

* The mean differences are significant at P < 0.05 as determined by Fischer’s protected LSD test

** The mean differences are highly significant at P < 0.01 as determined by Fischer’s protected LSD test

almost similar to those found in normal plant inoculated with *G. fasciculatum* (0.73%).

DISCUSSION

The benefits like increased growth performance,

disease resistance, improved water relation, reduced pathogenic infection and the improvement of soil structure by improving root health of the host plant have been accredited to the mycorrhizal association in plants (Jeffries, 1987).

Effect of mycorrhizal association on Growth Performance

In the present study, *G. mosseae* and *G. fasciculatum* were inoculated to one month old plantlets of normal and micropropagated plants

Table 4. Influence of AM fungal association on contents of chlorophyll after 4 months of treatment

Treatments	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
Normal	0.12 ±0.02	0.06±0.03	0.13 ±0.03
Normal <i>G. mosseae</i>	0.13 ±0.02*	0.06 ±0.02	0.15 ±0.02
Normal <i>G.fasciculatum</i>	0.14 ±0.02*	0.06 ±0.04	0.15 ±0.03
Micropropagated	0.15 ±0.02*	0.08 ±0.04*	0.21 ±0.04*
Micropropagated <i>G. mosseae</i>	0.16 ±0.03*	0.09 ±0.03*	0.21 ±0.04*
Micropropagated <i>G. fasciculatum</i>	0.17 ±0.03*	0.11 ±0.02**	0.22 ±0.02*

* The mean differences are significant at $P < 0.05$ as determined by Fischer's protected LSD test

** The mean differences are highly significant at $P < 0.01$ as determined by Fischer's protected LSD test

of *A. paniculata* to study the effects of symbiotic mycorrhization on morphology and drug content. These two species of AM fungi were selected because of their availability and known efficacy of endomycorrhization with micropropagated plants (Fortuna et al., 1992). Both normal and micropropagated plants showed good colonization with AM fungi. However, the highest percentage of colonization and sporulation were recorded in micropropagated plants, especially those inoculated with *G. fasciculatum*. This indicates the effectiveness of AM fungi in the better establishment of micropropagated plants. Endomycorrhization during the weaning stages of micropropagated plants results in more efficient root system which helps in the better uptake of phosphorus and other nutrients. Among the two AM fungi, *G. fasciculatum* showed better colonization with both normal and micropropagated plants than *G. mosseae*. *Glomus fasciculatum* was found to be good symbionts for medicinal plants as reported by Karthekeyan et al. (2009). However, Arpana and Bagyaraj (2007) have observed an improved growth of normal plants of *A. paniculata* inoculated with *G. mosseae* and *Trichoderma harzianum* either alone or in combination. However, Chiramel et al., (2006) have reported that *G. intraradices* and *G. leptotichum* are the best symbionts for normal plants of *A. paniculata*. Thus, the extent of improvement by AM fungi varied with the species of AM fungi and depends on their capacity to take up P from soil and transfer it to the host. Host specificity of AM fungi also well documented by Smith et al. (2000) and Burleigh et al. (2002). Hence, selection of efficient AM fungi plays a significant role in achieving maximum benefits from the association.

In the present study, there was a positive correlation between percent mycorrhizal colonization and plant growth. All the morphological parameters assessed were significantly influenced by AM fungal association both in normal and micropropagated plants. As there was good

percent of colonization in micropropagated plants, the growth was more pronounced than normal plants which were reflected in the increase of biomass. The increase in biomass was two fold as compared to normal control plants where the fresh and dry weights were found to be 16.5 g and 6.85 g respectively. Pope et al. (1983) had observed 200 percent increase in total dry weight in *Platanus occidentalis* inoculated with *G. fasciculatum*. Symbiotic association of *G. fasciculatum* with micropropagated plants of *Sesbania sesban* (Subhan et al., 1998) and a few selected medicinal plants had improved the biomass of mycorrhizal plants (Karthikeyan et al., 2009). The increase in biomass can be attributed to well developed root system in AM inoculated plants which help in better uptake of both nutrients and water from the soil (Sowmya et al., 2004). Mycorrhizal fungi may be able to influence host growth by the production of hormones as reported by Cooper (2000). Allen et al. (1980) detected increased cytokinin activity in mycorrhizal plants than non mycorrhizal plants.

The aforesaid discussion has highlighted the host specificity of AM fungi which has influenced the growth performance of the host plants. *Glomus fasciculatum* treated micropropagated plants of *Andrographis paniculata* showed improved biomass compared to other treatments in the present study.

Effect of Mycorrhizal association on Chlorophyll content

Mycorrhizal association improved the chl-a, chl-b and total chlorophyll contents in both normal and micropropagated plants. Positive correlation was observed between enhanced biomass and chlorophyll contents. Micropropagated plants inoculated with *G. fasciculatum* recorded the highest percent of colonizations and increased biomass was found to con-

Table 5. Influence of AM fungal association on contents of primary metabolites after 4 months of treatment

Treatments	Total carbohydrates (µg/g)			Reducing sugars (µg/g)			Total proteins (µg/g)			Total free amino acids (µg/g)		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Normal	453.20 ±0.37*	561.30 ±0.74*	447.20 ±1.06*	195.70 ±0.83*	215.60± 0.58*	189.30 ±0.54*	475.80 ±0.74	520.70 ±0.12	527.90 ±0.49	489.20 ±0.07	490.60 ±0.20	510.70 ±0.58
Normal <i>G. mosseae</i>	427.60 ±0.58	546.70 ±0.48	415.20 ±0.67	147.80 ±0.67	190.90 ±0.83	155.60 ±0.63	518.60 ±0.86*	538.60 ±1.36*	540.90 ±0.92*	496.80 ±0.37*	490.90 ±0.16	520.20 ±0.50*
Normal <i>G. fasciculatum</i>	432.00 ±0.63	530.00 ±0.86	406.80 ±0.37	168.40 ±1.04	198.50 ±0.37	160.20 ±0.80	530.90 ±0.35*	543.80 ±0.53*	549.60 ±0.56*	490.80 ±0.67	514.30 ±0.93*	589.00 ±0.40*
Micropropagated	487.70 ±0.24**	576.80 ±0.91**	468.00 ±0.54**	220.40 ±0.40**	231.70 ±0.37**	199.60 ±0.48*	550.90 ±0.63*	578.90 ±0.26*	639.30 ±0.33*	509.10 ±0.59*	539.40 ±1*	549.60 ±0.50*
Micropropagated <i>G. mosseae</i>	418.00 ±0.31	550.80 ±0.86	425.80 ±0.20	195.60 ±0.63*	210.70 ±0.50*	170.20 ±0.48	608.70 ±0.16*	615.60 ±0.87*	665.70 ±0.59*	520.50 ±0.58*	559.90 ±0.36*	560.70 ±0.46*
Micropropagated <i>G. fasciculatum</i>	428.30 ±0.89	479.90 ±1.20	445.20 ±0.78	230.20 ±0.58**	218.40 ±0.97*	229.60 ±0.40**	650.80 ±0.28**	620.80 ±0.11**	689.40 ±0.07**	578.40 ±0.28**	614.90 ±0.43**	629.60 ±0.63**

* The mean differences are significant at P < 0.05 as determined by Fischer's protected LSD test

** The mean differences are highly significant at P < 0.01 as determined by Fischer's protected LSD test

Table 6. Influence of AM fungal association on secondary metabolites after 4 months of treatment

Treatments	Total phenolics (µg/g)			Andrographolide (%)
	Root	Stem	Leaf	Leaf
Normal	410.30 ± 0.37	398.60 ± 1.26	389.10 ± 0.60	0.61 ± 0.007*
Normal <i>G. mosseae</i>	428.50 ± 0.50*	440.60 ± 0.96*	392.10 ± 0.73	0.67 ± 0.006*
Normal <i>G. fasciculatum</i>	430.70 ± 0.50*	433.40 ± 0.15*	400.80 ± 0.37*	0.73 ± 0.006*
Micropropagated	440.80 ± 0.50**	413.40 ± 0.37*	407.10 ± 0.44*	0.72 ± 0.002*
Micropropagated <i>G. mosseae</i>	420.70 ± 0.44*	450.80 ± 0.80**	457.70 ± 0.38**	0.65 ± 0.006*
Micropropagated <i>G. fasciculatum</i>	485.40 ± 0.89**	469.50 ± 0.04**	459.60 ± 0.76**	1.05 ± 0.005**

* The mean differences are significant at P < 0.05 as determined by Fischer's protected LSD test

** The mean differences are highly significant at P < 0.01 as determined by Fischer's protected LSD test

tain more chl-a&b and total chlorophyll than *G. mosseae* inoculated normal and micropropagated plants. Taiz and Zeiger (1998) correlate the enhancement of chlorophyll to Cu uptake since it is involved in the electron transport system and a component of chlorophyll-proteins. Enhancement in the rate of synthesis of chlorophyll may be a consequence of stress by either abiotic or biotic factors. Though AM fungal colonization results in symbiosis, the association might exerts a sort of stress on the host. AM fungi inoculated plants of *Lactuca sativa* under salt stress condition had higher photosynthetic capacity because of high chlorophyll content than those of non stressed plants indicating the counterbalance of salt stress by mycorrhization (Zuccarini, 2007). In *Bacopa monnieri* chlorophyll content was more in micropropagated plants inoculated with *G. mosseae* (Sowmya et al., 2004). However, in *Artemisia annua*, chlorophyll contents remained same in both control and AM inoculated plants (Kapoor et al., 2008). An increase in the chlorophyll content in the leaves of the mycorrhizal plants generally leads to the enhancement in the rate of photosynthesis which is reflected in the better growth performance as seen in the present and other studies.

Effect of mycorrhizal association on primary and secondary metabolism

Primary metabolites are considered as building blocks and needed for general growth and primary physiological function and are also the precursors of secondary metabolites. A typical feature of AM symbiosis is that the fungus depends upon the host for the supply of photosynthates from the roots of host plants. Owing to the heterotrophic nature of AM endophytes, the carbohydrate status of the host plant ought to change when root system sustain the fungus growth. Mycorrhizal plants differ from non mycorrhizal plants in the significantly higher C translocation from the shoot to root system (Snellgrove et al., 1982). The roots of AM inoculated normal and micropropagated plants had less carbohydrate than the control plants. Whereas, the reduced sugars content in the roots and leaves of *G. fasciculatum* inoculated micropropagated plant was more than the control. The loss of photosynthate through the root system is compensated by greater assimilation in the leaves of micropropagated plants. However, in *G. mosseae* inoculated plants, the contents of reduced sugars were less than the control in both normal and micropropagated plants. The variation in reducing sugars in normal and micropropagated plants in response to different AM fungi indicate the host specificity exhibited by AM fungi.

The comparison between normal and micropropagated plants was made with regard to the primary metabolite contents. Micropropagated plants both control and AM inoculated plants showed significant

increase suggesting a direct correlation with the better synthetic machinery, higher chlorophyll content and more foliar area as recorded in micropropagated plants (Tejavathi and Rao, 1998). Further, contents of total proteins and amino acids were significantly high in both normal and micropropagated plants inoculated with *G. fasciculatum*. France and Reid (1983) had attributed the increase in amino acids and protein to reverse translocation of carbon compounds to the host. The carbon compounds forms amino acids resulting from the incorporation of ammonium into carbon skeletons derived from fungal trehalose and mannitol. Lewis (1975) has suggested that the carbon moves from host to fungus as carbohydrates could return back to host as other compounds, through catabolism which provide necessary skeletal material for transfer of amino acids between fungus and host. Cooper (2000) is of the opinion that changes in the contents of reduced sugars and amino acids depends on the P status of the host plant. However, Matsubara et al. (2009) have attributed the increase in amino acid contents in strawberry plants in response to *G. mosseae* to some unknown factors other than effect of mycorrhizal association on secondary metabolism. In terms of cellular economy, the heterogenous secondary metabolites accumulate in less quantity than primary metabolites. Most of these compounds have been selected by means of evolutionary process with relatively potent biological activity (Balandrin and Klocke, 1988).

There are several reports on the effect of AM fungal association on secondary metabolism in host plants (Peipp et al., 1997; Kapoor 2008; Morone – Fortunato and Avato, 2008). Plant phenolics, including flavonoids are best studied for their role on effective symbiosis and plant defensive system (Carlsen et al., 2008; Perner et al., 2008). The increased resistance of AM fungal roots against pathogens and nematodes could be associated at least in part with marked host metabolic changes including enhanced production of phenolic compounds (Dehne, 1982). Phenolics are known to be of major importance in pathogenic interactions between plants and fungi. Increased phenolic concentration in plant tissues following pathogen attack is one of the important mechanism by which pathogen activity may be limited or decreased (Morandi, 1996). Munzenberger et al. (1990) have reported accumulation of cell bound phenolics in mycorrhiza inoculated roots of a few selected gymnosperms. *Glomus fasciculatum* inoculated micropropagated plants contained more soluble phenolics than other treatments. Accumulation of phenolic compounds during early stages of colonization may reflect the defense response of the plant against fungal penetration. Apart from phenolics, colonization of AM fungi in plants may lead to marked systematic reactions in terpenoid metabolism. Peipp et al. (1997) have reported two types of metabolism in AM fungal inoculated barley – one is phenolics and other one is

terpenoid metabolism. They concluded that accumulation of phenolic compounds in the early stages of infection may reflect the defense response, whereas the terpenoid metabolism as found in several members of Poaceae may be related to mycorrhizal formation as reported by Maier et al. (1997). The AM fungi induced accumulation of terpenoids in the members of Poaceae was compared to phytoalexin concept and thought to be a metabolic perturbations caused by the penetration of AM fungi (Kuc, 1995). *G. intraradices* induces accumulation of significant amount of leaf sesquiterpenoids in *Citrus jambri* (Nemec and Lund, 1990). The sesquiterpenoid ABA reaches considerably higher level in *Glomus* colonized maize than the control plants indicating the influence of AM fungus on the enzymes involved in terpenoid biosynthesis (Danneberg et al., 1993). Inoculation with *G. mosseae* enhanced the accumulation of the triterpens in cucumber roots, while no accumulation was observed by inoculating with the fungal pathogens (Akiyama and Hayashi, 2002). Andrographolide, abicyclic diterpenoid lactone is the main active principle of *A. paniculata*. The HPLC analysis of methanolic extracts of AMF inoculated normal and micropropagated plants along with control plants showed the presence of Andrographolide. However, the content was more in micropropagated plant inoculated with *G. fasciculatum* which contain 1.05% (w/w) of Andrographolide compare to normal control plants which recorded 0.61% (w/w) of active principle. The differences in the contents of Andrographolide in normal and micropropagated plants inoculated with two different species of AM fungus may be related to the compatible association between the hosts and specific AM fungus. Since, the primary metabolites like amino acids and proteins are the precursors for secondary metabolism; one can expect the increase in aminoacids and protein contents as a result of AM fungal symbiosis generally leads to enhanced secondary metabolism. Thus the micropropagated plants of *A. paniculata* inoculated with *G.fasciculatum* had shown enhanced primary and secondary metabolism resulted in the accumulation of more quantities of active principle-Andrographolide than other samples.

CONCLUSION

It can be concluded based on the aforesaid results, *Glomus fasciculatum* proves to be a good symbiont with the micropropagated plants of *Andrographis faniculata* which has reflected in good growth performance, increased primary and secondary metabolism in the host.

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