Arbuscular Mycorrhizal Technology for the Growth Enhancement of Micropropagated *Spilanthes acmella* Murr.

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Abstract

Yadav K., Singh N., Aggarwal A. (2012): **Arbuscular mycorrhizal technology for the growth enhancement of micropropagated** *Spilanthes acmella* Murr. Plant Protect. Sci., **48**: 31–36.

Spilanthes acmella (Asteraceae) is an endangered ornamental cum medicinal annual herb. This study reports on the effect of two arbuscular mycorrhizal fungi (*Glomus mosseae* and *Acaulospora laevis*) applied either alone or in combination on post-transplanting performance of micropropagated *S. acmella.* Complete plantlets of *S. acmella* were raised by direct organogenesis from nodal explants on Murashige and Skoog medium supplemented with various cytokinins. *S. acmella* plantlets responded to all three mycorrhizal treatments in a significantly different way. *G. mosseae* enhanced the survival rate of *S. acmella* plantlets to 100%. Plant height, plant spread, number of branches per plant, number of leaves per plant, leaf area, biomass production, and chlorophyll content were significantly higher in AM inoculated plantlets as compared to the uninoculated ones. This clearly demonstrates that AM inoculation not only improved the survival rate of micropropagated plantlets but also their growth and biomass yield.

Keywords: *Glomus mosseae*; *Acaulospora laevis*; *Spilanthes acmella*; acclimatisation**;** micropropagation

Abbreviations: AMF – Arbuscular mycorrhizal fungi; AM – Arbuscular mycorrhizal

Spilanthes acmella Murr. (family: Asteraceae), commonly known as "Akarkara" or "toothache plant", is a valuable ornamental cum medicinal herb occurring in the tropics and in subtropical parts of the world (Jansen 1981). In India, it has been reported from South India, Chattishgarh and Jharkhand (Anonymous 1989) and from Morni hills in Haryana (Singh 1995). The plant has immense application in pharmaceuticals, food, and health and body care products as flowers and leaves have been used as a spice for appetisers and as folk medicine for stammering, toothache, stomatitis, and throat complaints (Nakatani & Nagashima 1992). It also possesses anti-inflammatory, antibacterial and antifungal properties due to the presence of a highly valuable biologically active compound spilanthol (Khadir *et al.* 1989; Pandey *et al.* 2009). Because of its multifold uses, *S. acmella* is being overexploited by the local population as well as pharmaceutical companies. It is an acutely threatened plant species (Rao & Reddy 1983).

In micropropagation, the growth substrate is devoid of microbes and, as a result of this nutrient-rich growth substrate and delicate plants having no interaction with microorganisms (Dolcet-Sanjuan *et al.* 1996). Although micropropagation is an established technique for the production of elite plants, owing to transient transplantation shock, plants require biological hardening before transplantation. For this reason, mycorrhizal technology can be applied to reduce transplantation shock during acclimatisation, thus increasing plant survival and establishment rates (Estrada-Luna *et al.* 2000; Rai 2001).

Nowadays, Arbuscular mycorrhizal (AM) fungi are recognised as biofertilisers (Lovato *et al.* 1996), biological control of root pathogens (Reddy *et al.* 2006), bioremediation (Li *et al.* 2006), enhancing plant growth (Parkash *et al.* 2005), protection against toxicity (Aggarwal *et al.* 1999), soil fertility (Charles *et al.* 2006), drought tolerance (Wilson *et al.* 1991), salt tolerance (Sheng *et al.* 2009) and they also improve the productivity of medicinal compounds (Karthikeyan *et al,* 2009).

The benefits associated with the use of AM inoculation for *in vitro* raised plantlets have been reported in several horticultural and forest tree species (Rai 2001). However, little is known about the influence of AM fungi on survival and growth of micropropagated medicinal plant species (Gaur & Adholeya 1999; Sharma *et al.* 2008; Yadav *et al.* 2011).

During the past few years, considerable efforts have been made for *in vitro* plant regeneration of this valuable plant species (Saritha & Naidu, 2008; Singh *et al.* 2009; Yadav & Singh 2010). In order to fulfil the demands of pharmaceutical industries, it is necessary to develop an efficient method of *S. acmella* propagation. Thus, a greenhouse study was conducted to determine whether AMF inoculation could enhance the survival and post-transplantation performance of micropropagated plants of *S. acmella* and thus helping in the conservation and sustainable utilisation of this endangered herb for medicinal purposes.

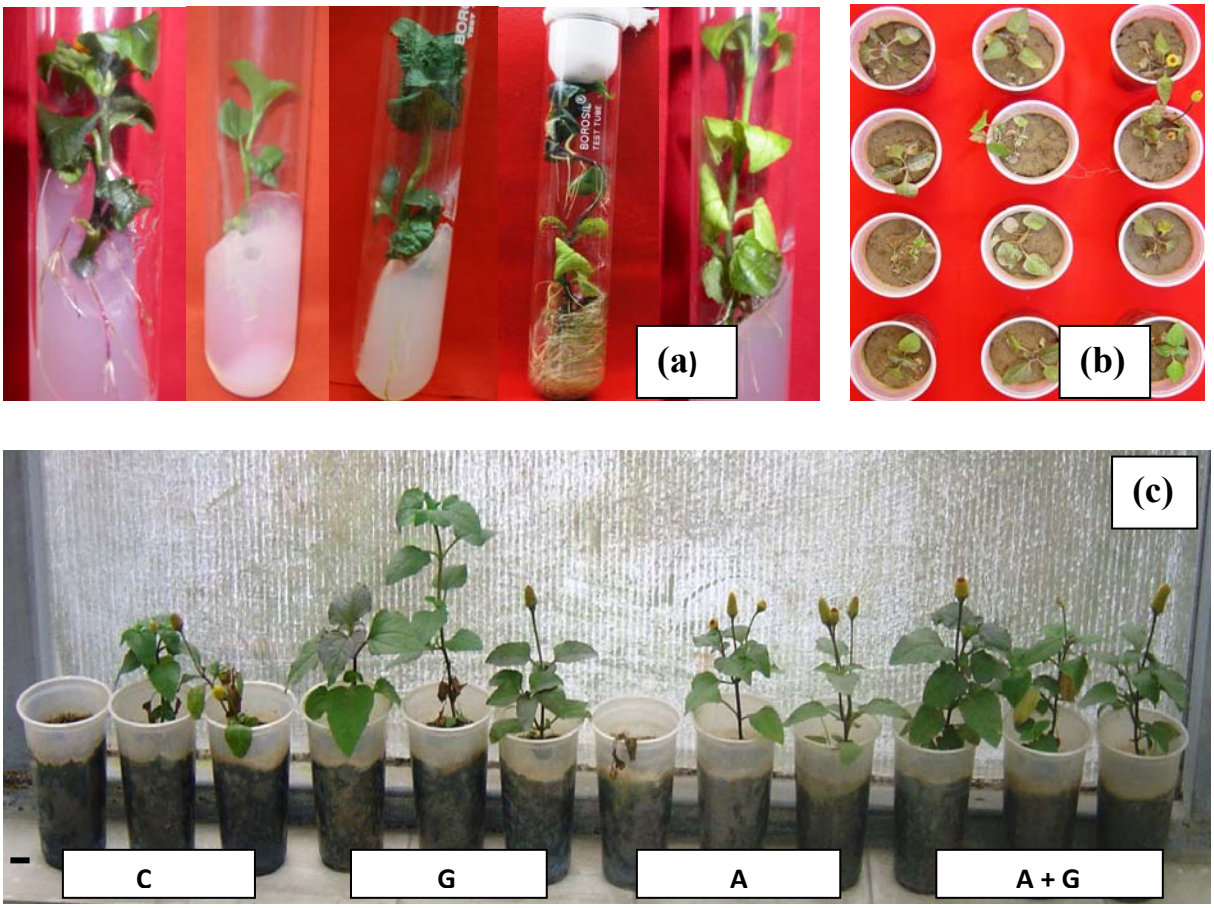
MATERIAL AND METHODS

Plant material and micropropagation procedures. *Spilanthes acmella* plants were collected in Ch. Devilal herbal garden, Chuharpur, Yamuna-Nagar, Haryana (India) and transplanted to pots in the Polyhouse of Botany Department, Kurukshetra University, Kurukshetra (India). Nodal segments (1–1.5 cm in length) of these plants served as a source of explants for micropropagation. Complete plantlets of *S. acmella* consisting of well-developed roots and leaves were obtained by direct organogenesis of nodal explants on MS medium (Murashige & Skoog 1962) containing 30 g/l sucrose and 8 g/l agar following the modified protocol by Yadav and Singh (2010). All cultures were maintained in a culture room at 25 ± 2°C under a photoperiod of 16 h light and 8 h dark cycle with a light intensity of 40 μmol/m^2/s^2 provided by cool white fluorescent lamps with 55–60% relative humidity until well-developed root formation occurred. The rooted micropropagated plantlets were removed from the agar medium and transplanted in plastic cups (14 × 8 cm) containing a sterilised soil and sand mixture (3:1), i.e. 375 g of soil and 125 g of sand, having different treatments with AM fungi for acclimatization. The cups were covered with transparent polythene bags (to maintain humidity) with holes (to provide aeration) and kept in a culture room initially for 15 days under the same light and temperature conditions provided during micropropagation. After 15 days these plantlets were transferred in the polyhouse for further studies.

Choice of AM fungus. Two dominant AM fungi (*G. mosseae* and *A. laevis*) as reported by Gupta *et al.* (2009) were isolated from the rhizosphere of *Spilanthes acmella* using a wet sieving and decanting technique described by Gerdemann and Nicolson (1963). The purpose of using an AM fungus alone and in combination was to assess which one is better for acclimatization and for various growth parameters.

Multiplication of AMF cultures. Both AM fungi were mass multiplied in sterilised sand and soil (1:3) substrate using maize as a suitable host in polyhouse conditions. The starter inoculum or pure culture of each selected dominant AM fungus was raised the funnel technique of Menge and Timmer (1982) using maize as a host. In this technique, the sterilised soil and sand mixture (3:1) was used as a substrate and filled in the funnel up to the neck portion. Two or three spores of AM fungi, i.e. *G. mosseae* or *A. laevis*, were introduced into the funnel mixture. Then the seeds of maize were sown in the funnel and watered regularly. This complete system including seedlings having AM colonised roots and soil samples containing AM spores were transferred to bigger pots containing a sterilised soil to sand (3:1) mixture for further multiplication of individual AM spores, using maize as a trap plant again.

Inoculation. *In vitro* raised plants (Figure 1a) were taken out from the culture tubes and washed in sterilised distilled water. Further the roots of those plants were washed with the help of a fine brush to remove the agar particles. These rooted plants were then transplanted in cups containing sterilised sand and soil (3:1), i.e. 375 g of soil and 125 g of sand. To each cup, 10% inoculum consisting of AM spores and fine roots of maize



C – Control; G – *Glomus mosseae;* A – *Acaulospora laevis*

Figure 1. (a) Micropropagated *Spilanthes acmella* in plant tissue culture Lab.; (b) Plants after 15 days of inoculation; (c) Plants after 45 days of inoculation

having mycelium/arbuscules/vesicles alone and in combination were added.

Growing conditions. The AM inoculated plants were grown in a greenhouse for acclimatisation, where maintained humidity was approximately 70% and temperature was 25–30°C. Light was provided by cool white fluorescent lamps (8000 lux) under a 16-h photoperiod. The polyhouse also received sunlight (Figure 1b).

Five replicates were taken with the following combinations of treatments:

T1 – uninoculated, i.e. autoclaved sterile soil (control),
T2 – inoculated with *A. laevis*,
T3 – inoculated with *G. mosseae*,
T4 – inoculated with *G. mosseae* and *A. laevis*.

Plant growth parameters like plant height, plant spread, number of branches per plant, number of leaves per plant, leaf area, plant dry weight and chlorophyll content were recorded after a 90-day interval commencing from the day after transplanting (Figure 1c). Two such observations were made during the entire growth period of the plant. Plant spread was measured along the north-south and east-west direction, leaf area was measured using a leaf area meter (Leaf Area Meter 211; Systronics Ltd, Ahmedabad, India).). The content of chlorophyll (*a*, *b*. and total) was estimated by the formula of Arnon (1949). Plant dry weight was determined after drying the tissue in an oven at 80°C for 48–72 hours.

Statistical analyses. All results were analysed using analysis of variance (ANOVA), followed by post hoc test with Statistical Package for Social Sciences (SPSS, version 11.5). Means were ranked at a $P \leq 0.05$ level of significance using Duncan's Multiple Range Test for comparison.

RESULTS AND DISCUSSION

Though there are reports on beneficial effects of AM fungi on various crops, a literature search

Table 1. Influence of AM fungi on the survival rate of micropropagated *Spilanthes acmella* after 90 days of inoculation

Treatments		Mortality rate (%)
T1	Control	32.0 ± 10.95[d]
T2	A	16.0 ± 8.94[c]
T3	G	0[a]
T4	G+A	8.0 ± 10.95[b]
LSD ($P \leq 0.05$)		11.99
ANOVA ($F_{3,8}$)		11.667*

Each value is a mean of five replicates; A – *Acaulospora laevis*; G – *Glomus mosseae*

Mean values followed by different letters within a column do not differ significantly from one another at $P \leq 0.05$ lead by Duncan's Multiple Range Test

*$P \leq 0.05$

reveals only a very few reports on the AM fungi association with micropropagated medicinal plants (Gaur & Adholeya 1999; Joshee *et al.* 2007; Sharma *et al.* 2008; Yadav *et al.* 2011).

In the present investigation, the *G. mosseae* inoculated plantlets showed 100% survival rate and better growth than non-mycorrhizal plantlets, but the magnitude of response varied according to the mycorrhizal treatment applied (Table 1).

Inoculation with *G. mosseae* + *A. laevis* significantly increased plant height as compared to uninoculated plants (Table 2). Similar results were also obtained in *Acorus calamus* (Yadav *et al.* 2011). Inoculation with *A. laevis* also resulted in taller plant height compared to uninoculated plants. The plant spread and leaf number per plant were higher when inoculated with *G. mosseae*, followed by the combination of *G. mosseae* + *A. laevis.* This may also be due to increased uptake of nutrients via the increased absorbing surface of AM inoculated plants. High mortality in micropropagated plantlets is primarily due to a poorly developed or non-functional root system (Clapperton & Reid 1992; Wang *et al.* 1993).

Leaf area per plant was also significantly larger in inoculated treatments as compared to uninoculated treatments. The plant inoculated with *G. mosseae* possesses a larger leaf area. Treatment with *A. laevis* took a smaller number of days for flowering as compared to treatments with *G. mosseae* + *A. laevis* or *G. mosseae* alone (data not shown). Similar trend was observed in the number of branches per plant (Table 2 and Figure 1c). Larger leaf area in AM inoculated plants might be as a result of enhanced phosphorus acquisition.

The plants inoculated with *G. mosseae* had significantly higher biomass as compared to other treatments. An increase in plant biomass because of AMF inoculation was also reported in *Scutellaria integrifolia* (Joshee *et al.* 2007)

The increased growth of mycorrhizal plantlets in terms of plant height and leaf area than that of non-mycorrhizal plantlets might be due to enhancement in the anabolic processes (especially

Table 2. Influence of AM fungi on the various growth parameters of micropropagated *Spilanthes acmella* after 90 days of inoculation

Treatments	Plant height (cm)	Leaves (no./plant)	Leaf area (cm^2/plant)	Branches (no./plant)	Plant spread (cm^2)	Fresh weight (g/plant)		Dry weight (g/plant)	
						shoot	root	shoot	root
T1 control	22.0 ± 3.16[b]	38.0 ± 4.47[c]	16.18 ± 2.50[c]	8.6 ± 0.89[c]	525.2 ± 15.20[c]	8.42 ± 0.20[c]	1.04 ± 0.012[b]	1.68 ± 0.15[c]	0.20 ± 0.01[c]
T2 A	23.4 ± 3.97[b]	46.4 ± 6.84[b]	23.44 ± 2.01[b]	15 ± 0.70[a]	554.0 ± 21.82[c]	12.19 ± 1.52[b]	2.08 ± 0.009[a]	2.47 ± 0.20[b]	0.34 ± 0.02[b]
T3 G	28.0 ± 3.16[a]	57.6 ± 7.12[a]	36.64 ± 1.74[a]	10.2 ± 1.30[b]	753.6 ± 33.06[a]	18.60 ± 1.29[a]	1.48 ± 0.025[ab]	3.44 ± 0.11[a]	0.26 ± 0.02[c]
T4 G+A	30.4 ± 2.61[a]	49.6 ± 9.31[ab]	30.68 ± 2.35[a]	12.4 ± 1.67[b]	680.0 ± 44.47[b]	16.19 ± 0.93[a]	2.44 ± 0.14[a]	3.07 ± 0.15[a]	0.43 ± 0.03[a]
LSD ($P \leq 0.05$)	4.3754	9.5842	2.986	1.6145	41.2048	1.5088	0.2243	0.2152	0.0346
ANOVA ($F_{3,8}$)	7.216*	6.433*	79.280*	26.609*	60.919*	76.761*	68.672*	115.033*	75.216*

Each value is a mean of five replicates; A – *Acaulospora laevis*; G – *Glomus mosseae*

Mean values followed by different letters within a column do not differ significantly from one another at $P \leq 0.05$ lead by Duncan's Multiple Range Test

*$P \leq 0.05$

Table 3. Effect of different treatments with AM fungi on chlorophyll concentration (mg/100 g) in the leaves of micropropagated plantlets of *Spilanthes acmella* after 90 days

Treatments		Chlorophyll *a* (mg/100 g f. wt)	Chlorophyll *b* (mg/100 g f. wt)
T1	Control	0.045 ± 0.00[d]	0.204 ± 0.03[d]
T2	A	0.074 ± 0.00[c]	0.292 ± 0.01[c]
T3	G	0.212 ± 0.04[a]	0.524 ± 0.06[a]
T4	G+A	0.170 ± 0.01[b]	0.424 ± 0.05[b]
LSD ($P \leq 0.05$)		0.0526	0.0888
ANOVA ($F_{3,8}$)		28.433*	25.285*

Each value is a mean of five replicates; A – *Acaulospora laevis*; G – *Glomus mosseae*
Mean values followed by different letters within a column do not differ significantly from one another at $P \leq 0.05$ lead by Duncan's Multiple Range Test
*$P \leq 0.05$

photosynthesis) as a result of better uptake and mobilization of various essential nutrients and water (Panwar 1991). The leaf chlorophyll content recorded in the mycorrhizal plants was typically higher than in the non-treated control. The maximum total chlorophyll content was noticed in *G. mosseae* inoculated plants, followed by those inoculated with both fungi (Table 3). These results are in accordance with the results obtained by Sharma *et al.* (2008). AM treated plants allow the root system to exploit a greater volume of soil by extending away the root zone and by exploring smaller soil pores not reached by the root hairs. The improved soil texture that occurs increases air and water percolation and facilitates the root system access to soil water and nutrients.

Improved growth and survivability of mycorrhizal plantlets in this study may be due to an increase in nutrient uptake from soil. This technology may also be applied to improve the post-transplanting performance of some other medicinally important plants which require conservation efforts.

Acknowledgements. The authors are thankful to the Kurukshetra University, Kurukshetra for providing infrastructure facilities to carry out the work. Financial support from U.G.C., New Delhi is gratefully acknowledged.

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Received for publication April 30, 2011
Accepted after corrections September 19, 2011

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