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Mycorrhization of Micropropagated *Leucaena leucocephala* (Lam.) de Wit

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Abstract

The influence of arbuscular mycorrhiza (AM) and *Rhizobium* during *ex vitro* hardening stage on growth and survival of micropropagated *Leucaena leucocephala* was investigated. *Ex vitro* inoculation with the AM fungus *Glomus fasciculatum* plays a key role in favoring increased growth and survival of these plants. The dual inoculation of *G. fasciculatum* and *Rhizobium* has resulted in higher growth rate of root and shoot, number of leaves, number of nodules, mycorrhizal colonization and 100% survival of micropropagated plants up to 5 months as compared to 91.66% and 65.21% survival in only mycorrhizal and *Rhizobium* treated plants, respectively. The non-inoculated plants grew poorly and all the plants died after 5 months. This biotechnology therefore, appears to be of potential interest in ensuring improved growth and survival of micropropagated multipurpose legume trees during the nursery stage.

Keywords: Arbuscular mycorrhiza, *Rhizobium*, *Leucaena leucocephala*, micropropagation, *ex vitro* inoculation

1. Introduction

Leucaena leucocephala (Lam.) de Wit is one of the most important multipurpose legume trees both from the ecological and the economic point of

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view for its use as food, fuel, fodder and afforestation in tropics. It is difficult to propagate this plant by conventional methods of propagation. Efforts have been made for *in vitro* nodulation of micropropagated *Leucaena leucocephala* by *Rhizobium* strain NGR 8 (Dhawan and Bhojwani, 1986). However, the hardening process was not only expensive and time consuming but was also inadequate for the survival of micropropagated plants (Dhawan and Bhojwani, 1987). Efforts have not been made so far to explore the association of AM fungi – a vital natural symbiotic component of *Leucaena leucocephala* – during its micropropagation studies. In nature AM fungi benefit plants essentially by improving mineral nutrition, water uptake and hormone production (Pate, 1994; Marschner and Dell, 1994). Mycorrhizal association has been investigated in a number of currently micropropagated plants (Varma and Schuepp, 1994; 1996; Guillemin et al., 1992; 1994; Vidal et al., 1992; Branzanti et al., 1992).

The application of AM fungi also provides an effective protection against fungal pathogens in micropropagated systems where plants are at even greater risk to pathogens due to poor cuticular and root development (Guillemin et al., 1994; Vestberg et al., 1994; Hooker et al., 1994). *L. leucocephala* is normally colonized by AM fungi in the field and growth enhancement has been obtained by inoculating AM fungi on seedlings (Huang et al., 1985; Habte and Manjunath, 1987; Osonubi et al., 1991). The present investigation was carried out to find out the effect of separate and dual *ex vitro* inoculation of *Glomus fasciculatum* and *Rhizobium* strain PRGL 001 on growth and survival of micropropagated plantlets of *L. leucocephala*.

2. Materials and Methods

Micropropagation of host plant

Cotyledonary nodes (1 cm long segments) were excised from 15 day old seedlings and cultured on modified B_5 medium (Gamborg et al., 1968) supplemented with 6-benzylamino purine (BAP) 5×10^{-6} M for multiple shooting. Rooting of individual shoots was obtained in half strength B_5 medium supplemented with Indole 3-butyric acid (IBA) at concentration of 5×10^{-6} M. The cultures were maintained at 28±2°C with a light/dark cycle of 12/12 hours. Light was supplied from fluorescent tubes at 200 uE/m^2/s intensity. The plantlets obtained were acclimatized in bottles containing sterilized soilrite with one-fourth strength of liquid B_5 medium. After three weeks, the plants were transplanted to polythene bags containing sterilized soil with one-tenth strength of liquid B_5 medium (Kumari and Pardha Saradhi, 1992). The soil used in the experiment was sandy with pH 7.7,

organic carbon 0.36%, EC 0.8 mmhos/cm, available P 45 kg/ha, available K 705 kg/ha, Zn 2.46 ppm, Cu 0.78 ppm, Fe 4.92 ppm, and Mn 8.28 ppm.

Mycorrhizal inoculation

Spores of *G. fasciculatum* (Thaxter sensu Gerd.) Gerd and Trappe were isolated from Delhi ridge soil and multiplied in pot culture containing sterilized Delhi ridge soil on roots of *Trigonella* sp. for four months. The inoculum consisted of mycorrhizal roots with the extramatrical hyphae and soil containing spores. Ten gram of inoculum (25 spore/g soil) was applied to each plantlet close to the root system while transferring from soilrite to polybags containing fumigated soil.

Rhizobial inoculation

Rhizobium strain (PRGL 001) procured from Division of Microbiology, IARI, New Delhi was maintained and multiplied on Yeast extract Mannitol Agar (YEMA) medium. Rhizobial inoculation was done at 2 stages – when the plants were transferred to autoclaved soilrite and also at the time of transfer of plants to fumigated soil. Each time about 2 ml rhizobial suspension (ca. 10^4 cells/ml) was added to soilrite and to fumigated soil, respectively.

Growth conditions

Inoculated and non-inoculated plants were grown on fumigated soil in a polyhouse under controlled conditions at 30±2°C, and RH 55–60%. The plants were watered thrice a week. No fertilizer or nutrient solution was supplied.

Experimental setup

Four different treatments were given to micropropagated plants: i) control (uninoculated); ii) inoculated with *G. fasciculatum;* iii) inoculated with *Rhizobium;* and iv) inoculated with *Rhizobium* and *G. fasciculatum*. Growth parameters like root and shoot length, number of nodules, number of leaves, root and shoot dry weight and survival percentage of plants were recorded at 30, 90, and 150 days. An average of five readings was taken.

Mycorrhizal status

Mycorrhizal colonization in roots was assessed at weekly intervals according to the method of Phillips and Hayman (1970).

Statistical analysis

Student t-test was applied to determine the level of significance of differences between means of treated and control plants (Mead and Curnow, 1983).

3. Results

All the growth parameters viz. root and shoot length and their dry weight, number of leaves, number of nodules, mycorrhization and survival of the plants were significantly influenced with *ex vitro* mycorrhizal inoculation.

Plant growth responses

In dual (*G. fasciculatum* + *Rhizobium*) inoculated plants, the root length was significantly ($P \leq 0.01$) higher as compared to uninoculated control after 30 days of transplantation in soil. Single inoculation of either mycorrhizal fungus or *Rhizobium* did not result in significant increase of root length during one month. However, after 90 days, a highly significant ($P \leq 0.001$) increase in root length was observed with dual inoculation and mycorrhizal inoculation alone (Table 1). The plants with dual inoculation assumed consistently maximum height followed by single inoculation of mycorrhiza and *Rhizobium* over control. In dual inoculated plants, the shoot length was 69.9% and 64.1% higher at 30 and 90 days, respectively as compared to control and was significant at ($P \leq 0.001$) level. The inoculation of *G. fasciculatum* was also effective in inducing better growth than only *Rhizobium* inoculation. Accordingly the biomass of dual inoculated plants and inoculated with mycorrhiza alone was significantly ($P \leq 0.001$) higher than only *Rhizobium* and control. After 90 days, the increase in dry weight of dual inoculated plants was more than 7 fold whereas in only mycorrhizal inoculated plants the increase was more than 6 times over the control (Table 1).

The dual inoculated plants developed lush green leaves and the number of leaves increased with the age of the plants while other treatments did not show any significant difference in number of leaves after one month. The leaves in control plants grew poorly, developed chlorosis and started shedding after one month (Table 1).

Nodulation and mycorrhization

The nodule development gradually increased with the growth of treated plants. The nodule formation was higher in dual inoculation than in either of

Table 1. Effect of different treatments on growth parameters of micropropagated *L. leucocephala* at three different time periods.

Growth parameter	Treatment	Days after transplant in soil 30	90	150
Root length, cm	Control	4.8 ± 0.44	7.18 ± 0.89	–
	G. fasciculatum	6.16 ± 1.88	13.8 ± 1.88***	29.7 ± 1.56
	Rhizobium	5.76 ± 1.4	11.88 ± 1.91**	21.2 ± 0.71
	Rhizobium + G. fasciculatum	11.95 ± 3.82**	15.32 ± 1.08***	31.8 ± 6.99
Shoot length, cm	Control	4.86 ± 0.64	5.52 ± 0.38	–
	G. fasciculatum	6.52 ± 1.01*	8.64 ± 0.81***	19.1 ± 1.74
	Rhizobium	6.44 ± 0.69**	6.92 ± 1.07*	16.26 ± 0.23
	Rhizobium + G. fasciculatum	8.26 ± 0.45***	9.06 ± 0.57***	21.94 ± 2.76
Root dry weight, g	Control	0.011	0.032 –	
	G. fasciculatum	0.017	0.214 ± 0.081**	1.024 ± 0.069
	Rhizobium	0.013	0.053 ± 0.02	0.79 ± 0.241
	Rhizobium + G. fasciculatum	0.021	0.235 ± 0.056***	1.99 ± 0.727
Shoot dry weight, g	Control	0.039 ± 0.015	0.099 ± 0.116	–
	G. fasciculatum	0.062 ± 0.043	0.645 ± 0.06***	2.572 ± 0.944
	Rhizobium	0.045 ± 0.011	0.123 ± 0.116	1.393 ± 0.405
	Rhizobium + G. fasciculatum	0.080 ± 0.036	0.789 ± 0.212***	5.688 ± 2.00
Number of leaves	Control	3.0 ± 0.55	1.8 ± 0.45	–
	G. fasciculatum	3.4 ± 0.55	4.6 ± 1.14**	12.0 ± 0.84
	Rhizobium	3.4 ± 0.89	3.2 ± 0.45**	8.4 ± 1.14
	Rhizobium + G. fasciculatum	5.2 ± 0.45***	6.4 ± 1.14***	18.8 ± 3.11
Number of nodules	Control	–	–	–
	G. fasciculatum	–	6.6 ± 2.3***	7.6 ± 1.1
	Rhizobium	1.4 ± 0.55***	17.2 ± 5.81***	25.4 ± 1.14
	Rhizobium + G. fasciculatum	3.8 ± 3.49	19.4 ± 4.72***	29.6 ± 3.71

Values are expressed as mean ± SD, where n = 5. *'t' is significant at P≤0.05; **'t' is significant at P≤0.01; ***'t' is significant at P≤0.001.

the single inoculations. However, single *Rhizobium* inoculation induced 3.34 fold more nodules than only mycorrhizal inoculation at 150 days (Table 1).

Mycorrhization was also increased with the age of the plants. The plants inoculated with *G. fasciculatum* showed 9.52% root colonization in the form of external hyphae and appressoria after one week of inoculation whereas it was delayed for two weeks in dual inoculated plants. Percent mycorrhization was higher in *G. fasciculatum* inoculated plants as compared to dual inoculated plants up to 4 weeks. After 4 weeks, mycorrhization was *at par* in both the treatments (Fig. 1). Internal hyphae and arbuscules were observed after 2 weeks and vesicles were observed only after 4 weeks of inoculation.

Survival of plants

The survival of micropropagated plants was remarkably improved with mycorrhizal inoculation. The improved growth of root, shoot and leaves and significant nodulation and mycorrhization resulted in 100% survival of dual inoculated plants throughout the experiment as compared to 91.66% and 65.21% survival in only mycorrhizal and *Rhizobium* inoculated plants, respectively. Uninoculated plants grew poorly and their survival rate decreased sharply from 82.14% at 30 days to 14.28% after 90 days and ultimately 100% mortality was observed after 150 days of transplant (Fig. 2).

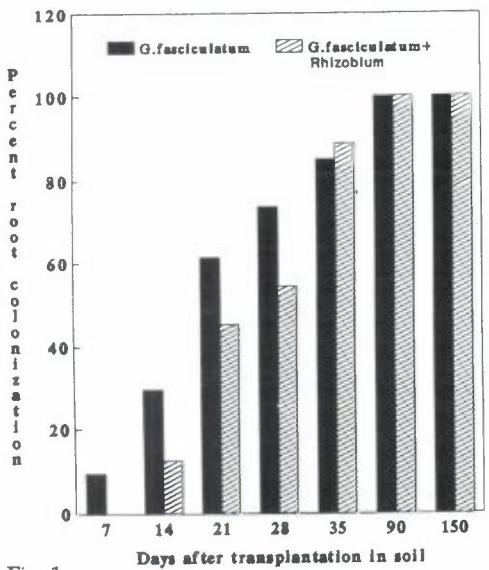

Fig. 1.

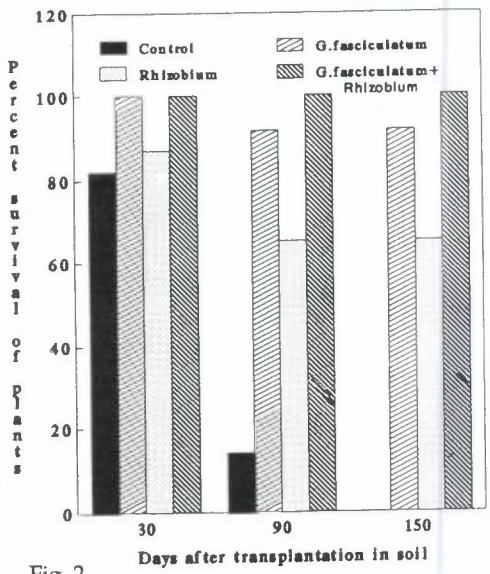

Fig. 2.

Figure 1. Percent mycorrhization of micropropagated *L. leucocephala* in different treatments.

Figure 2. Percent survival of micropropagated *L. leucocephala* in different treatments.

4. Discussion

Recognizing the importance of mycorrhizal association in plant growth, the technique of mycorrhizal inoculation in micropropagated horticultural plants like apple (Branzanti et al., 1992), avocado (Vidal et al., 1992), Kiwi fruit (Schubert et al., 1990, 1992), pineapple (Guillemin et al., 1992, 1994), plum (Fortuna et al., 1992), strawberry (Varma and Schuepp, 1994, 1996; Vestberg 1992a, 1992b) has been widely used and *ex vitro* mycorrhizal inoculation has been reported to increase growth and survival of plants. In the present investigation, *ex vitro* inoculation of AM fungi has resulted in significant increase in root and shoot growth, their dry weight, number of leaves and survival of the plants as compared to control. The mechanism of enhancement of growth and total survival of micropropagated *L. leucocephala* seems to be due to the ability of arbuscular mycorrhiza to increase plants nutrient uptake from the soil similar to established field grown plants (Marschner and Dell, 1994; Smith et al., 1994). Inoculation with AM fungi appears to be critical for the survival and growth of micropropagated plants (Salamanca et al., 1992; Sivaprasad et al., 1995). Increase in growth following mycorrhization has been attributed to improvement in P availability to the host through solubilization of P by the release of phosphatase enzymes (Gianinazzi-Pearson and Gianinazzi, 1976, 1978; Tarafdar and Marschner, 1994; Thiagarajan and Ahmad, 1994) and to additional effects such as modifications in hormonal balance by mycorrhizal symbiosis (Allens et al., 1980, 1982). After 30 days of inoculation, active mycorrhization resulted in renewed apical growth whereas shoot apices of uninoculated plants failed to resume activity which resulted in stunted growth, chlorosis, leaf shedding and subsequent death of all control plants after 5 months. Mycorrhizal inoculation of micropropagated plants can also produce modifications in root morphology and dynamics which could help in the establishment and growth of plantlets (Berta et al., 1990; Hooker et al., 1992). In mycorrhizal *L. leucocephala* greater root density was observed due to profuse development of lateral roots which resulted in 568.7% increase in root dry weight at 90 days as compared to control. However, no significant difference between mycorrhizal and non-mycorrhizal roots occurred at 30 days after transplant. Similar increased production of lateral roots of *in vitro* micropropagated *Vitis vinifera* was observed when inoculated with *G. fasciculatum* at outplanting (Schellenbaum et al., 1991).

The synergistic effect of *Rhizobium* and AM fungi has been established in many field grown plants (Valdes et al., 1993; Ishac et al., 1994; Saito and Kato, 1994; Vyas and Srivastava, 1995). *Rhizobium* strain (PRGL 001) and *G. fasciculatum* isolated from Delhi ridge soil were found highly compatible in the present tripartite symbiotic system of *Leucaena leucocephala*. Higher

mycorrhizal colonization and nodulation in dual inoculated plants have resulted in maximum growth and survival of micropropagated plants as compared to single inoculation of either symbionts. Increased growth and survival may be attributed to the synergistic effect of both the symbionts. Mycorrhizal colonization augments P concentration in plant tissue which in turn stimulates nodulation activity for increased N_2 fixation. Growth stimulation of dual inoculated plants may be explained by improved uptake of N and P in tissues. Similar results have been obtained in dual inoculation of AM fungi and *Rhizobium* in field grown legume trees (Cruz et al., 1988; Dixon et al., 1993; Manjunath et al., 1984), *Vicia faba* (Ishac et al., 1994) and in *Phaseolus vulgaris* (Daniels-Hylton and Ahmad, 1994). Apart from the effect on the host's P supply, there is also a possibility that arbuscular mycorrhiza could influence the legume-*Rhizobium* symbiosis by altering the rhizosphere environment for rhizobia (Crush, 1974). Results of the present study indicate that the *ex vitro* dual inoculation of *G. fasciculatum* and *Rhizobium* may be used successfully as cost effective and eco-friendly technique in mass multiplication of *L. leucocephala* during nursery stage for improved growth and survival of micropropagated plants and may further be tested for other multipurpose legume trees.

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