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Effect of mycorrhizal inoculation on growth and establishment of micropropagated *Leucaena leucocephala* (Lam.)de Wit

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Abstract

The effect of mycorrhizal inoculation during ex vitro hardening stage on growth and survival of micropropagated plants was studied using Leucaena leucocephala as test plant. Ex vitro inoculation with Arbuscular Mycorrhizal Fungus (AMF) Glomus macrocarpum plays an important role in improved growth, establishment as well as survival of micropropagated plants. Plants inoculated with Glomus macrocarpum showed significant increase in all plant growth parameters. Root length ,shoot length ,number of leaves ,plant biomass significantly increased due to mycorrhizal inoculation. Percent survival was also considerably influenced by mycorrhizal inoculation .After 150 days, the survival percent was zero in uninoculated control as compared to 90% in mycorrhizal plants .An early inoculation of micropropagated plants with AM fungi will ensure improved growth and survival of micropropagated forest trees during nursery stage to be used in reforestation programs .It is desirable to inoculate the tissue-cultured plantlets with suitable arbuscular mycorrhizal fungi as early as possible before nursery production for raising the survival rate after transplantation and also stimulating the growth of tissue cultured plantlets.

Keywords: *Leucaena leucocephala* ,Arbuscular mycorrhiza, micropropagation.

1. Introduction

Leucaena leucocephala (Lam.) de Wit is a tree of high commercial value as forage and firewood and is particularly employed in reforestation programs in developing countries.

This multipurpose tree is a nitrogen -fixing legume that helps to enrich soil. Its ability to thrive on steep slopes, in marginal soils and in areas with extended dry seasons makes it a prime candidate for restoring forest cover, slopes and grasslands that have been denuded through wood cutting or fire.

It is difficult to propagate this plant through traditional methods. Among techniques of plant biotechnology, micropropagation is cited as the most successful example which has become an important commercial industry. This technique is used as a research tool as well as for commercial plant production. Nowadays micropropagation is the most widely and successfully used technology by private companies for mass production of ornamentals, vegetables, fruits, plantation crops, spices. One major benefit for utilising micropropagation techniques in plant production is the capacity to implement the breeding programs rapidly with large numbers of cloned micropropagated plants being produced in relatively short time. Secondly, micropropagated plantlets are usually free of diseases due to their initiation from meristems and maintenance in axenic conditions. Application of tissue culture technique for regeneration and propagation of forest trees for tree improvement programs is becoming a promising area of research. One of the greatest challenges which technique of micropropagation should meet is to identify and overcome factors which affect the survival and establishment of micropropagated plants after their transfer from culture conditions to the greenhouse or field conditions .During acclimatization stage, plants are subjected to severe environmental stress due to poor root, shoot and cuticular development .This results in high losses due to low survival rate as well as large increase in fertilizer and pesticide chemical inputs Due to their economic and ecological importance, the leguminous trees constitute a significant component of forest vegetation. The regeneration rate of these trees in natural surroundings is quite low, therefore methods for rapid in vitro micropropagation and genetic improvement are required. Many workers have made efforts to develop plantlets of ***Leucaena leucocephala*** *in vitro*. But hardening process was inadequate for the survival of micropropagated plants [1, 2]. Tissue cultured plants are susceptible to transplantation shocks which leads to high mortality during the final stage of micropropagation. Technique of tissue culture results in elimination of useful microbes like Arbuscular mycorrhiza, which are an integral part of natural ecosystem. Thus, during relatively long periods of their development the plants are without Arbuscular mycorrhizal fungi, and later on also due to their transfer of such plants into inert potting media or fumigated nursery beds. However, many studies have demonstrated the importance of early inoculation of these tissue cultured plants to avoid adverse effects such as stunting of seedlings in fumigated soil or poor growth and development after transplanting [3, 4]. The benefits of mycorrhizal application in plantlets have been demonstrated in several herbaceous and woody species such as guava [5], date palm [6], fruit trees [7,8], ***Vitis vinifera*** [9], avocado [10] cassava[11]. However, scientific reports on the influence of AM fungi on growth and survival of micropropagated forest tree species are limited. Mycorrhizae is mutualistic association formed by most species in the plant kingdom, including almost all the plants which are currently micropropagated [12]. Arbuscular mycorrhizal fungi (AMF) belonging to phylum Glomeromycota symbiotically associate with the plant roots [13]. During last two decades, there have been many studies to explore the association of AM fungi_ an important natural symbiotic component of plants in natural ecosystem_ in micropropagated systems. In nature AM fungi provide number of benefits to plants. Increase in uptake of soil phosphorus by mycorrhizal fungi is well documented [14]. Not only phosphorus, but uptake of several other poorly mobile micronutrients like copper, zinc, iron ,manganese are increased in presence of AM fungi. The extramatrical hyphal network of AM fungi take up nutrients from the soil and translocate them to the internal mycelium where they are released into the root cells [12]. Mycorrhizae have a potential to biologically suppress the root pathogens like ***Phytophthora*** sp., ***Fusarium*** sp., ***Rhizoctonia*** sp. as well as nematodes [15].

Leucaena leucocephala is normally colonized by AM fungi in the field and increased growth has been

obtained by inoculating AM fungi. The present study highlights the effect of *ex vitro* inoculation of ***Glomus macrocarpum*** on growth and survival of micropropagated plants of ***L. leucocephala*** following transplantation.

2. Materials and Method

Micropropagation of host plant

Seeds of ***Leucaena leucocephala*** were treated with 20% sulphuric acid for 1 hr and soaked at 80°C for 6 hr in distilled water. Seeds were surface sterilized using 0.1% $HgCl_2$ for 3-4 minutes. Thereafter all the subsequent steps were carried out in a laminar flow chamber under aseptic conditions. The seeds were washed thrice with sterilized distilled water, and germinated axenically on 0.8% agar.1 cm long segments of cotyledonary nodes were excised from 15 day old seedlings and cultured on modified B_5 medium [16] supplemented with 6-benzylaminopurine (BAP) 5×10^{-6} M. Multiple shoots obtained were excised and used for root induction. Rooting of individual shoots was done in half strength B_5 medium supplemented with Indole-3-butyric acid (IBA) at 5×10^{-6} M. The cultures were maintained at 28 ± 2°C with a light/dark cycle of 12hr/12hr. Light was supplied from fluorescent tubes at 200 $\mu E/m^2/s$ intensity. The plantlets thus obtained were transferred to bottles containing sterilized soil rite for acclimatization and given one-fourth strength of liquid B_5 medium. After 3 weeks the plants were subsequently transplanted to polythene bags containing sterilized soil and covered with polythene bag to maintain high humidity for about 1 week. The plants were given one-tenth strength of liquid B_5 medium at the time of transplantation in soil.

Mycorrhizal inoculation

Glomus macrocarpum was used for inoculating the tissue cultured plants. Spores were multiplied in pot culture on roots of *Trigonella* sp. for four months. The inoculum consisted of mycorrhizal roots with the external mycelium as well as soil containing spores. 10g inoculum was applied to each plantlet close to the root system. The rooted plantlets were inoculated when transferred from soil rite to polythene bags containing fumigated soil.

Growth conditions

Inoculated and non-inoculated (control) plants were grown in a polyhouse under controlled conditions with temperature ranging from 30 ± 2°C, and relative humidity around 55-60%. No fertilizer or nutrient solution was supplied to plants during the course of study. Plants were watered daily with tap water.

Experimental setup

Micropropagated plants were given following treatments:

1. Control (uninoculated).
2. Inoculated with ***G. macrocarpum.***

Different growth parameters like root length and shoot length, number of nodules, number of leaves, root and shoot dry weight as well as survival rate was recorded at 1month, 3 month, 5 month. An average of five readings was taken.

Mycorrhizal status

Mycorrhizal colonization in roots: The technique of Phillips and Hayman [17] was used.

3. Results and Discussion

Mycorrhizal inoculation showed significant effect on all plant growth parameters. After 30 days of transplantation in soil, inoculation with *G.macrocarpum* resulted in significant increase in root length as compared to control (Table 1). At 90 days, mycorrhizal plants showed highly significant increase in root length. Changes in root morphology of mycorrhized plantlets under water stress plays an important role in improved growth as number of lateral roots increase surface area for nutrient and water absorption Significant increase in shoot length was obtained in mycorrhizal plants at 60 days. Inoculation with *G. macrocarpum* did not result in any significant increase in number of leaves at 30 days but at 60 days, inoculated plants showed highly significant increase in number of leaves. At 90 days, root dry weight showed highly significant increase in

mycorrhizal plants whereas shoot dry weight was 3 times more in mycorrhizal plants as compared to control (Table 1). Mycorrhizal plants showed consistently good growth, lush green leaves. Control plants grew poorly, leaves developed chlorosis and started shedding after one month. Most of the researches regarding mycorrhizal inoculation in micropropagated system have been carried out in horticultural plants like plum, apple, kiwifruit, pineapple, strawberry, grapevine, raspberry, avocado. All the workers have reported an increased growth of micropropagated plantlets following ex vitro inoculation [5, -11, 18, 19]. In the present investigation, similar enhancement in growth has been observed in micropropagated *L.leucocephala* following mycorrhizal inoculation. Arbuscular mycorrhizal fungus increased growth to a great extent. The mechanism of growth enhancement and total survival of micropropagated plants seems to be due to the ability of arbuscular mycorrhiza to increase plant nutrient uptake from the soil similar to established field grown plants [20]. Biochemical changes brought about by mycorrhization were helpful in mitigating different stresses experienced by the tissue cultured plants during hardening, which determine their performance later in field [21].

Improved growth parameters after mycorrhizal inoculation has been attributed to improvement in P availability to the host through solubilization of P by the release of phosphatase enzymes [22] and to additional effects such as modifications in hormonal balance by mycorrhizal symbiosis [23] .Mycorrhizal inoculation of micropropagated plants can also produce modifications in root morphology and dynamics which could help in the establishment and growth of the plantlets. In micropropagated *Vitis vinifera*, similar increased production of lateral roots was observed in mycorrhizal plants at outplanting [9].

Table 1: Effect of mycorrhizal inoculation on growth parameters of micropropagated *L.leucocephala* at three different time periods. (Naqvi)

Growth Parameters	Treatments	Days after transplantation in soil				
		30		90		150
		Mean+	Observed 't' value	Mean+	Observed 't' value	Mean+
Root Length(cm)	Control	4.8 ± 0.44	-	7.18 ± 0.89	-	-
	Mycorrhiza	7.82 ± 2.27	2.63*	13.12 ± 1.67	6.32***	26.4 ± 0.61
Shoot Length(cm)	Control	4.86 ± 0.64	-	5.52 ± 0.38	-	-
	Mycorrhiza	5.96 ± 1.19	1.64	8.7 ± 0.5	10.23***	18.12 ± 2.42
Number of Leaves	Control	3.0 ± 0.55	-	1.8 ± 0.45	-	-
	Mycorrhiza	3.2 ± 0.71	0.44	3.6 ± 0.55	5.14***	9.2 ± 0.84
Root Dry Weight(g)	Control	0.011	-	0.032	-	-
	Mycorrhiza	0.018	-	0.173 ± 0.058	7.05***	0.844 ± 0.071
Shoot Dry Weight(g)	Control	0.0.39 ± 0.015	-	0.099 ± 0.116	-	-
	Mycorrhiza	0.055 ± 0.020	1.33	0.349 ± 0.096	3.57**	1.891 ± 0.565

+Values are expressed as mean ± SD where n=5.

*Observed 't' is significant at P ≤ 0.05.

**Observed 't' is significant at P ≤ 0.01

***Observed 't' is significant at P ≤ 0.001.

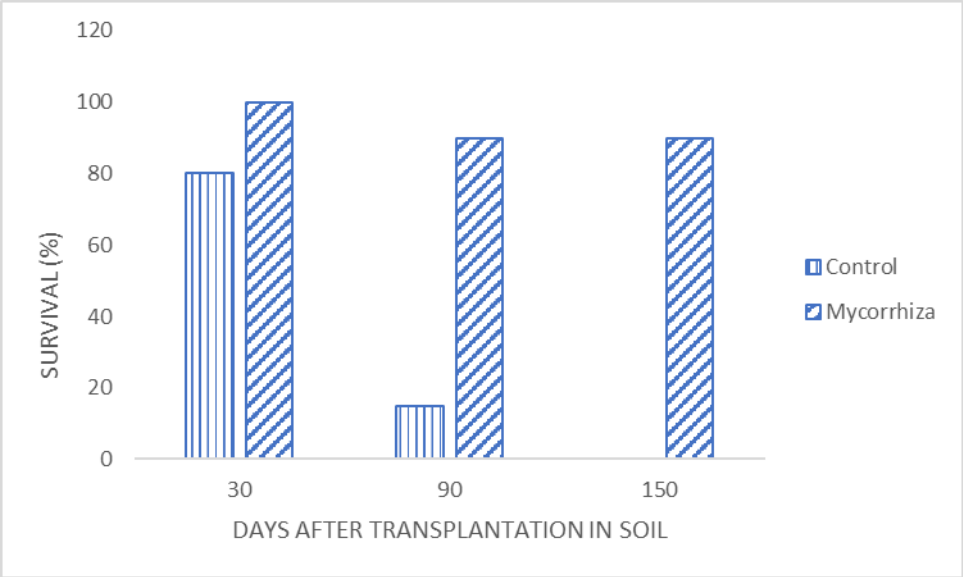

Fig. 1: Percent survival of micropropagated *L. Leucocephala* as influenced by mycorrhizal inoculation. (Naqvi)

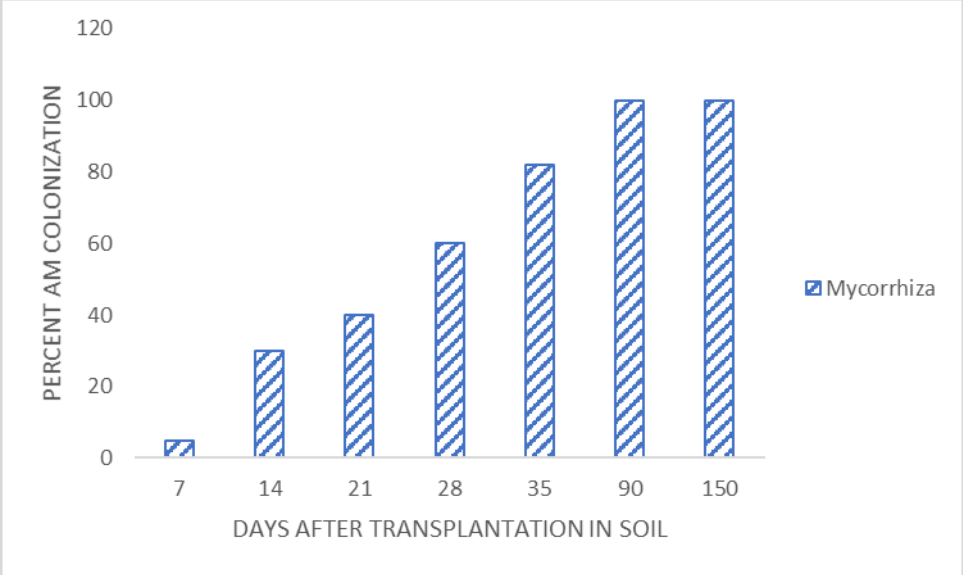

Fig. 2 Mycorrhizal root colonization (%) of micropropagated *L. leucocephala* at different time periods (Naqvi)

The number of surviving plants was recorded after 30, 90 and 150 days. The percent survival was considerably influenced by mycorrhizal inoculation (Fig.1). In mycorrhizal plants shoot apices were active with no apparent transplantation shock. Similar improvement in survival rate has been reported by many workers [24, 25, 26]. In contrast survival of non-mycorrhizal plants decreased with time with reduced number of apical buds. After 150 days, survival percent was zero in uninoculated plants as compared to 90% in plants inoculated with *G. macrocarpum* (Fig.1).

Mycorrhization increased with age of the plants. The mycorrhizal inoculated plants showed root colonization in the form of external hyphae and appressoria after one week of inoculation. At this time, hyphal entry points were observed only. Internal hyphae and arbuscules were observed after 2 weeks whereas vesicles were observed only after 4 weeks of inoculation. With elongation of the roots, new arbuscules were produced

behind the apex of lateral roots. At 90 days, the rate of roots colonized by AM fungi reached to 100% (Fig.2)

4. Conclusion

Mycorrhizal fungi can play very pivotal role in the improved growth, establishment as well as survival of micropropagated multipurpose legume trees used in forestry and revegetation programs. This environment friendly and cost-effective technique will give tremendous boost to commercial nurseries and will result in decrease fertilizer and pesticide inputs. Early mycorrhizal inoculation and colonization of tissue - cultured plantlets may reduce transplantation shock during acclimatization, thus increasing plant survival and establishment rates.

Conflict of interest

No conflict of interest influenced in this research.

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