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Root Colonization and Improved Growth Performance of Micropropagated *Terminalia bellerica* Roxb. Plantlets Inoculated with *Piriformospora indica* during *ex Vitro* Acclimatization

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ABSTRACT

Tissue culture raised plantlets of *Terminalia bellerica*, when inoculated with endophytic root fungus *Piriformospora indica* during their *ex vitro* acclimatization showed higher survival rate and improved overall growth. The fungus grew intracellularly and colonized roots of more than 80% of plantlets. Inoculated plantlets were significantly taller, had more leaves and greater total leaf area. Colonization of roots by the fungus promoted root growth and consequently caused an increase in biomass and total chlorophyll contents, suggesting better uptake of nutrients.

Keywords: biopriming, endophytic fungus, medicinal plant
Abbreviations: BAP, 6-benzyl amino purine; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog medium; **SH**, Schenk and Hildebrandt medium; **SRM**, standard rooting medium

INTRODUCTION

Micropropagation biotechnology has been widely applied for rapid clonal propagation of a large number of economically important plant species. However, its widespread use is restricted by the often high percentage of plants lost or damaged when transferred to *ex vitro* conditions (Gaur and Adholeya 1999). Plantlets or shoots that have been grown *in vitro* are continuously exposed to a unique microenvironment providing minimal stress and optimum conditions for plant multiplication. Such conditions induce structural and physiological abnormalities in the plants rendering them unfit for survival under *in vivo* conditions (Hazarika 2006). Therefore, in any micropropagation system acclimatization of plantlets to a greenhouse or field environment is an essential step because of the difference in micropropagation and the greenhouse environment. Several strategies have been suggested to improve the process of hardening and acclimatization to reduce mortality of plantlets during transplantation (Hazarika 2003). The most common approach to improve plant survival upon transfer to soil is their gradual adaptation to the *ex vitro* environment. Certain chemicals, environmental factors and microorganisms presensitize the cellular metabolism of plant. Upon exposure to stress, the pre-sensitized or 'primed' plants adapt better and faster than the non-primed plants (Conrath *et al.* 2002). The priming of *in vitro* propagules includes chemical, physical and biological approaches prior to and /or upon transplanting (Nowak and Shulaev 2003). Recently, microbial inoculants have been used to induce stress resistance in plant propagules both as *in vitro* co-culture and on transplanting (Herman 1996; Balla *et al.* 1997). A newly discovered cultivable endophytic fungus, *Piriformospora indica* (**Fig. 1**) has been characterized as a plant growth-promoting, root colonizing fungus (Varma *et al.* 1999). This axenically culturable fungus resembles arbuscular-mycorrhizae (AM) fungi in several functions and physiological characteristics and show improved performance and reduced losses under stress environments (Singh *et al.* 2000).

Micropropagation studies on *Terminalia bellerica* Roxb. (Family-Combretaceae), an important medicinal plant have been carried out (Roy *et al.* 1987; Bilochi 2001; Ramesh *et al.* 2005; Rathore *et al.* 2008) but with limited success in field establishment of tissue culture raised plantlets. The present study has successfully demonstrated better growth and survival during hardening and acclimatization of tissue culture raised *T. bellerica* plantlets inoculated with *P. indica*.

MATERIALS AND METHODS

Source of explant

In vitro shoot cultures of *T. bellerica* were established using cotyledonary and epicotyledonary node explants obtained from 15-days-old aseptically raised seedlings. For aseptic seed germination, seeds were collected from superior identified trees and surface sterilized with 0.15% $HgCl_2$ for 10 min and washed thoroughly with autoclaved distilled water 4-5 times. Sterilized seeds were aseptically inoculated on water agar (solid medium containing 0.8% agar only) for germination. Seed germination started within 4-5 days. Cotyledonary and epicotyledonary nodes were excised aseptically from 15-days-old seedlings and inoculated on SH (Schenk and Hildebrandt 1972) medium supplemented with 1.5 mg l^{-1} BAP (6-benzylaminopurine), 3.0% sucrose and gelled with 0.8% agar in culture tubes (25 × 150 mm, Borosil) each containing *ca.* 17.0 ml culture medium. Shoots started to proliferate within 21 days. Proliferating shoots were subsequently transferred on to fresh MS (Murashige and Skoog 1967) medium supplemented with 1.5 mg l^{-1} BAP for further growth and multiplication. Repeated subculture was done every 21 days on same culture medium to obtain a large number of shoots.

For *in vitro* rooting proliferated shoots, each measuring 3-4 cm were aseptically excised and transferred on standard rooting medium (SRM) containing 1/4th MS salts, 1.0% sucrose and 0.1 mg l^{-1} IBA in culture tubes stoppered with non-absorbent cotton plugs. The pH of the media was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} for 15 min. All the cultures were kept under controlled conditions of temperature (28 ± 2°C), light (45

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µmol m^{-2} s^{-2} for 16 h per day provided by white fluorescent tubes, Philips) and 50-60% air humidity.

Culture of *Piriformospora indica*

P. indica was maintained on Kaefer's medium (Kaefer 1977). The fungus was grown either on agar-gelled slants or in broth. Fungal discs obtained from previously growing cultures of *P. indica* were used to inoculate Kaefer's broth and slants. The cultures were incubated at 30 ± 2°C in dark. The broth cultures were additionally kept under constant shaking conditions (100 rpm). Growth on slants was observed after 15-20 days while in broth, the fungus grew within 10 days. The fungus was examined under 100 and 400X of microscope before used for experimentations.

Root colonization and acclimatization

Uniformly rooted plantlets immediately after their removal from agar-gelled medium were subjected to *in vitro* hardening in culture bottles containing autoclaved Soilrite™ and covered with polypropylene caps. The Soilrite™ was moistened with 1/4th MS salt solution. Bottles were kept in standard greenhouse conditions (28 ± 2°C, a regime of gradually reducing humidity from 70-45% and 400 µmol m^{-2} s^{-2} irradiance) and their caps were opened gradually.

For colonization of rooted plantlets of *T. bellerica* with *P. indica*, fungal inoculation was provided during *in vitro* hardening of plantlets. For this purpose, mycelium was harvested from broth cultures and washed several times with sterile distilled water. Mycelium (about 50 mg fresh weight per plant) was mixed with autoclaved Soilrite™. The Soilrite™-fungus mix (SF) was distributed in culture bottles and in each bottles one rooted shoot was implanted. Total 25 plantlets were inoculated for experimentation. Non-inoculated plants of similar size were also maintained on Soilrite™ as control. Thirty-days old plantlets were transplanted into polybags containing soil, Soilrite™ and farmyard manure (1: 1: 1) and were allowed to grow under nursery shade conditions. At the time of transplantation various growth parameters were recorded from treated and untreated samples from randomly selected individuals.

Plant growth measurement

The shoot length, leaf number and mean leaf area, root number and mean root length, fresh weight, dry weight, percent survival, percent root colonization and chlorophyll contents of inoculated and non-inoculated plants were recorded after 30 days of inoculation of fungus. For observations and data collection, 10 plant samples from each treatment were collected randomly.

Staining of root samples

Roots of *T. bellerica* were washed thoroughly in running tap water, cut into 1.0 cm pieces and boiled in 10% KOH solution for 5 min. Thereafter, the root pieces were washed 3-4 times with sterilized distilled water and treated with 1% HCl for 3-4 min before staining with 0.05% trypan blue in lactophenol (Phillips and Hayman 1970). The stained root segments were examined microscopically (X100). The roots of control plants were also processed in similar way.

Assessment of root colonization

The method proposed by Giovannetti and Mosse (1980) was followed for assessment of root colonization. The root pieces (1.0 cm) were selected at random from the stained samples and observed under microscope (X40). In all, 25 segments were examined under the microscope and percent colonization was calculated as follows:

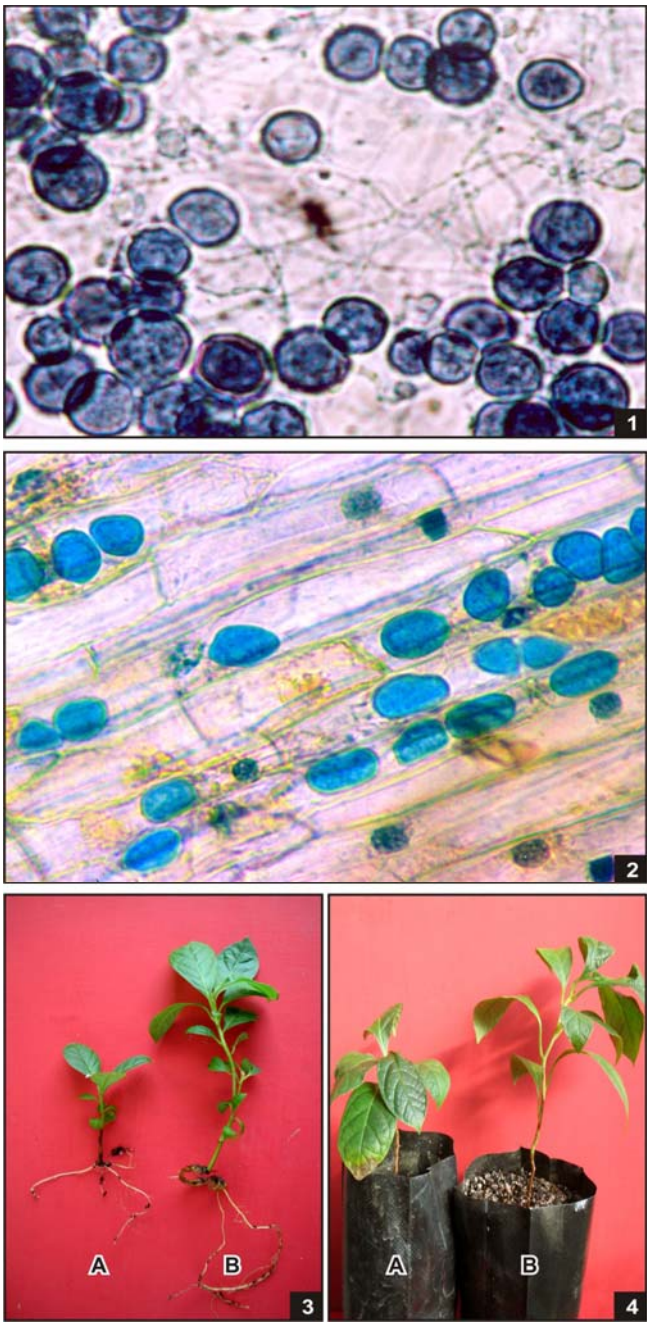
$$\text{Percent colonization} = \frac{\text{No. of root segments colonized}}{\text{Total no. of root segments examined}} \times 100$$

Chlorophyll content

Leaves from inoculated and non-inoculated plantlets were ground in excess chilled 80% acetone. Homogenized mixture was filtered through Whatman No. 1 filter paper. Absorbance of the filtrate was read at 663, 654 and 645 nm. The chlorophyll contents were calculated by using the formulae given by Arnon (1949).

Statistical analysis

The experiments were conducted in a completely randomized design (CRD). For each treatment six replicates were used. Experiments were repeated thrice and the mean values of one representative experiment have been presented. Data are presented as mean ± standard deviation and the difference between treatments was compared by Student's *t*-test.



Figs. 1-4 Root colonization of micropropagated *Terminalia bellerica* Roxb. plantlets inoculated with *Piriformospora indica* during *ex vitro* acclimatization. (**1**) Endophytic root fungus *P. indica* used during present investigation. (**2**) Root of *T. bellerica* colonized with *P. indica* showing chlamydospore in cortical cells. (**3**) Growth performance of tissue culture raised plantlets of *T. bellerica*. (**A**) Control (non-inoculated); (**B**) Inoculated with *P. indica*. (**4**) Growth performance under nurseryshed conditions. (**A**) Control (non-inoculated); (**B**) Inoculated with *P. indica*.

RESULTS AND DISCUSSION

In this work micropropagated plantlets of *T. bellerica* were inoculated with *P. indica* during *in vitro* hardening and overall growth performance and *ex vitro* survival of inoculated and non-inoculated plantlet were compared. Root colonization was observed after 10 days of inoculation where fungal mycelium grew intracellulary (**Fig. 2**). Consequently, a pronounced increment in overall growth and height of treated plantlets was recorded (**Figs. 3, 4**). The mean number of leaves (16.50 leaves) produced per plantlet was also higher in inoculated plantlets as compared to control (non-inoculated). Such leaves showed a mean area of 562.33 mm^2 per leaf which was also higher as against 313.33 mm^2 recorded for untreated control (**Table 1**). Significant increase in root length (10.10 cm) was recorded in treated plantlets as compared to non-inoculated plantlets (5.57 cm) which accounted for improved growth of such plantlets (**Table 2**). However, no significant difference in total number of roots was observed in treated and non-treated plantlets. The difference in growth observed between treated and control plantlets has been suggested to be caused by greater absorption of water and nutrients due to extensive colonization of roots by *P. indica* in *Spilanthes calva* and *Withania somnifera* under *in vitro* conditions (Rai *et al.* 2001). Varma *et al.* (1999) have reported similar increments in overall growth in *P. indica*-treated micropropagated plantlets of *Artemisia annua*, *Bacopa monnieri* and Tobacco. More intense root proliferation in these systems has been accounted to excessive synthesis of unidentified intracellular phytohormones by *P. indica* (Varma *et al.* 2001). *P. indica*-treated *T. bellerica* plantlets also showed an increase in total fresh (1178 mg) and dry (143 mg) weight in comparison to non-inoculated plantlets where 937 mg and 104 mg fresh and dry weight was recorded per plantlet, respectively (**Table 3**). Such an increase in biomass has been shown to be related to increased phosphate uptake by colonized roots (Sudha *et al.* 1998).

Microscopic examination of stained root samples revealed high colonization in more than 80% of plantlets. Colonization was observed intracellularly in epidermal and cortical cells where it formed coils, and branches or spores but typical arbuscules as generally developed by mycorrhizal association was not observed (**Fig. 2**). Root colonized plants on their transplantation in the polybags and grown under greenhouse conditions showed more than 90% survival as compared to 75% survival of untreated control plantlets (**Table 3**). High degree of plantlets survival during hardening and acclimatization of microprpagated plantlets of *Nicotiana tabacum* and *Bacopa monnieri* treated with *P. indica* has been reported (Varma *et al.* 1999). During the present investigation, the tissue culture raised inoculated plantlets registered significant increase in chlorophyll *a*, *b*, and total chlorophyll contents as compared to non-inoculated plantlets which might have contributed to high photosynthetic activity and consequent increase in overall growth and biomass (**Table 4**). The present studies have demonstrated that inoculation of micropropagated plantlets of *T. bellerica* with *P. indica* was beneficial both in term of overall growth and *ex vitro* survival in comparison to untreated control plantlets. Thus, present investigation highlights the role of *P. indica* as an excellent candidate for biological hardening of micropropagated plantlets of *T. bellerica*.

Table 1 Influence of *P. indica* root colonization on growth parameters of micropropagated plantlets of *T. bellerica* during hardening and acclimatization.

Treatment	Shoot length (cm)	№ of leaves	Leaf area (mm^2)
Non-inoculated	5.33 ± 0.40 b	9.50 ± 1.51 b	313.33 ± 1.21 b
Inoculated	8.75 ± 1.12 a	16.50 ± 1.37 a	562.33 ± 2.06 a
Significance (*t* test)	6.97**	8.36**	254.72**

All values are the mean ± SD; NS – mean values are not significantly different.
** mean values of treatments differ significantly at P ≤ 0.01

Table 2 Influence of *P. indica* root colonization on growth parameters of micropropagated plantlets of *T. bellerica* during hardening and acclimatization.

Treatment	№ of roots	Root length (cm)	Percent colonization
Non-inoculated	1.33 ± 0.51 b	5.57 ± 0.94 b	NA
Inoculated	1.66 ± 0.51 a	10.10 ± 0.86 a	87
Significance (*t* test)	1.12 (NS)	8.67**	

All values are the mean ± SD; NS – mean values are not differ significantly
** mean values of treatments differ significantly at P ≤ 0.01

Table 3 Influence of *P. indica* root colonization on some physiological parameters of micropropagated plantlets of *T. bellerica* during hardening and acclimatization.

Treatment	Fresh weight (mg)	Dry weight (mg)	Percent survival
Non-inoculated	937 ± 25.82 b	104 ± 8.90 b	75
Inoculated	1178 ± 109.64 a	143 ± 10.32 a	90
Significance (*t* test)	5.25**	7.09**	

All values are the mean ± SD; NS – mean values are not differ significantly
** mean values of treatments differ significantly at P ≤ 0.01

Table 4 Influence of *P. indica* root colonization on chlorophyll contents of micropropagated plantlets of *T. bellerica* during hardening and acclimatization.

Treatment	Chlorophyll contents (mg/g fresh tissue)		
	Chl ***a*** ± SD	Chl ***b*** ± SD	Total Chl ± SD
Non-inoculated	544 ± 5.55 b	843 ± 6.02 a	1386 ± 6.62 b
Inoculated	875 ± 17.63 a	767 ± 18.96 b	1655 ± 5.31 a
Significance (*t* test)	9.37**	43.87**	77.51**

All values are the mean ± SD; NS – mean values are not differ significantly
** mean values of treatments differ significantly at P ≤ 0.01

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