International Journal of Plant Developmental Biology ©2008 Global Science Books

Root Colonization and Growth Enhancement of Micropropagated *Feronia limonia* (L.) Swingle by *Piriformospora indica* – A Cultivable Root Endophyte

Shailendra Vyas • Rakhi Nagori • Sunil Dutta Purohit*

Plant Biotechnology Laboratory, Post Box No. 100, Department of Botany, Mohanlal Sukhadia University, Udaipur 313001, India

Corresponding author: * sdp_56@hotmail.com

ABSTRACT

In vitro-raised plantlets of *Feronia limonia* (L.) Swingle were colonized using an endosymbiotic root fungus *Piriformospora indica* during *in vitro* rooting and their *ex vitro* transfer. Improved growth has been observed in terms of higher shoot and root length, internode diameter, area and number of leaves and both fresh and dry weights in plants showing an association with this fungus. More than 90% of such plants survived in the greenhouse and subsequently under a nursery shed. The study demonstrated the potential of *P. indica* as a biopriming agent for achieving better growth and survival of tissue culture raised plantlets.

Keywords: biopriming, fruit tree, greenhouse growth, hardening and acclimatization

Abbreviations: BAP, 6-benzylaminopurine; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog medium; **SF**, Soilrite^{TM}–fungus mix; **SRM**, standard rooting medium

INTRODUCTION

In vitro propagation of plants is often associated with a high mortality rate during the *ex vitro* establishment phase. The *in vitro* conditions and culture room environment induce large number of variations at both macro and micro levels in micropropagated plants (Ziv 1991; Desjardins 1995; van Huylenbroeck and Debergh 1996). Consequently, when transplanted out of the culture vessel, they suffer from extreme environmental stress resulting in severe losses (Preece and Sutter 1991; van Huylenbroeck and Debergh 1996).

Manipulation of acclimation conditions has been reported to reduce losses, however, at an additional cost to the producer (Donnelly *et al*. 1985; Desjardins *et al*. 1988). Attempts have been made to reduce transplantation losses by the use of a CO_2-enriched environment and high light intensity for autotrophic micropropagation (Vyas and Purohit 2003; Dave and Purohit 2004). So far, this technology has yet to gain popularity (Nowak 1998). Microbial inoculants have been used to induce stress resistance in plant propagules produced *in vitro* prior to their transplantation (Herman 1996; Balla *et al*. 1997). Enhanced abiotic and biotic resistance in response to some microbial inoculant(s) leading to developmental and physiological changes in *in vitro* culture-derived plantlets has been referred to as 'biotization' (Herman 1996; Nowak 1998). Many plant beneficial, naturally associated algae, bacteria and fungi (especially arbuscular mycorrhiza fungi or AMF) have been shown to enhance stress resistance in micropropagated plants (Hooker *et al*. 1994; Elmeskaoui *et al*. 1995; Wilhelm *et al*. 1997; reviewed by Vosátka and Albrechtová 2008). Such biologically hardened plants show improved performance and consequently, reduced losses under stress environments.

Recently a cultivable endophytic fungus *Piriformospora indica* (**Fig. 1**) has been shown to improve the growth and overall biomass production in different herbaceous and tree species (Verma *et al*. 1998; Suthar and Purohit 2008). The *in vitro* grown shoots on treatment with *P. indica* have been reported to show early root differentiation and improved

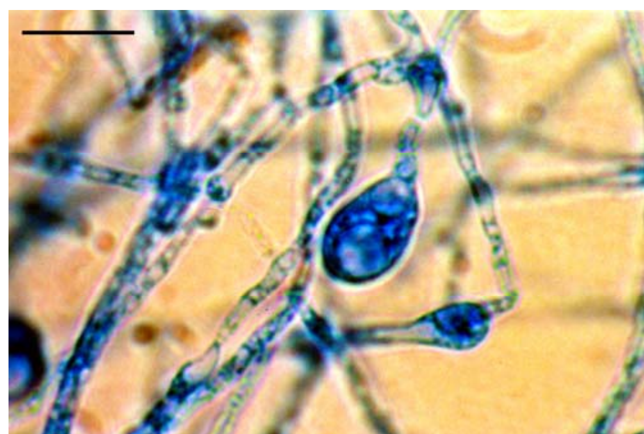

Fig. 1 Endosymbiotic root fungus *P. indica* used for biopriming. Bar = 10.0 μm.

drought tolerance (Varma *et al*. 2001). When transferred to pots, they showed a survival rate exceeding 90%.

Feronia limonia (L.) Swingle (Family: Rutaceae), commonly known as wood apple is a fruit tree of the Indian subcontinent and found widely distributed in the Aravalli ranges of south-east Rajasthan (Kirtikar and Basu 1993). Its superior genotypes are also cultivated for edible fruits, gum, wood and alkaloids. Fruits and other plant parts of *F. limonia* have been recommended for use against several ailments in the Ayurvedic system of medicine (Kirtikar and Basu 1993) and the presence of antimicrobial and antitumour compounds has been reported (Saima *et al*. 2000; Rahaman and Gray 2002). *In vitro* plantlet development in *F. limonia* has been reported using different pathways and a variety of explants (hypocotyl, epicotyl, cotyledonary leaf, nodal segments from seedling and mature plants) (Purohit and Tak 1992; Tak 1993; Hossain *et al*. 1994; Hiregoudar *et al*. 2003). Regeneration through adventitious shoot formation in *F. limonia* using hypocotyl segments was reported

Received: 31 July, 2008. Accepted: 19 September, 2008.

Original Research Paper

by Vyas *et al*. (2005). In the above reports, no attempts were made, however, to study the influence of *P. indica* on growth and *ex vitro* survival of *in vitro* raised plantlets. Therefore, in this investigation tissue culture-raised plantlets of *F. limonia* were colonized with *P. indica* in order to improve growth and to define the role of this fungus as a biopriming agent.

MATERIALS AND METHODS

Source of explants

In vitro shoot cultures of *F. limonia* were established using hypocotyl segments obtained from 20-days-old aseptically raised seedlings. Fresh seeds of *F. limonia* were obtained from a ripe fruit (collected from an identified superior tree) and rinsed in running tap water to remove all residual pulp, dried and stored for further use. For aseptic seed germination, seeds were surface sterilized with autoclaved solution of 0.1% $HgCl_2$ for 5 min, washed thoroughly with autoclaved double distilled water 4-5 times and inoculated aseptically on 0.8% agar (HiMedia Laboratories, India) dissolved in water in culture tubes (25 × 150 mm, Borosil make). Seeds started to germinate within 7-8 days.

In vitro regeneration and plantlet development

For *in vitro* culture establishment 1.5 cm-long hypocotyl segments were excised aseptically from 20-days-old seedlings and inoculated on Murashige and Skoog (1962) basal medium containing 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and kinetin (Kn) each and supplemented with 0.8% agar and 3.0% sucrose for adventitious shoot bud regeneration as per the protocol described by Vyas *et al*. (2005). Shoot induction was achieved within 45 days. The further elongation and multiplication of shoots was achieved on MS medium containing 0.5 mg l^{-1} BAP, 1.5 mg l^{-1} Kn, and 0.01 mg l^{-1} α-napthalene acetic acid. The explants were subcultured every 21 days on the same fresh medium. Shoots (*ca*. 3 cm long) were harvested from the multiplying cultures and inoculated on 0.8% agar–gelled standard rooting medium (SRM) containing 1/4 MS salts, 1.0% sucrose and 1.5 mg l^{-1} indole-3-butyric acid (IBA) for rooting. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C, 1.06 Kg cm^{-2} pressure for 15 min. All the inoculations for the purpose of *in vitro* culture of *F. limonia* were done aseptically on a Laminar Flow Clean Air Bench (Yorco, India). The cultures were maintained at 28 ± 2°C under a 16-h photoperiod having 45 µmol m^{-2} s^{-1} illumination provided by cool white fluorescent tubes (36 W, Philips).

Culture of *P. indica*

P. indica (DSM 11827) Verma, Varma, Kost, Rexer and Franken (Verma *et al*. 1998), kindly provided by Prof. A. Varma, was maintained on Kaefer 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 broth and slants. The cultures were incubated at 30 ± 2°C in the 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 (to confirm lack of contamination) under 100 and 400X before being used in the experiment.

Root colonization

For studying the effect of *P. indica* on root induction, fungus growing of Kaefer slants was used. To aseptically inoculate the basal cut ends of shoots derived from *in vitro* cultures of *F. limonia* as described in preceding sections, the multiplying shoots were cut using a scalpel blade. The shoot was held with a pair of forceps and the cut end of the shoot was touched once on the growing slants of *P. indica*. The growth of *P. indica* on SRM was not intended. The inoculum of *P. indica* was left adjacent to the cut ends of shoots for two purposes- one to see if the fungus could enhance root induction and second to observe simultaneous root induction and infection. The shoots (three to five shoots per flask) were then placed in SRM for root induction. Control shoots (without fungal treatment) were also placed on SRM for comparison. In all, 50 shoots were inoculated of which 25 were treated with *P. indica* and remaining 25 were kept untreated. For colonization of rooted plantlets of *F. limonia* with *P. indica*, fungal inoculation was provided during *in vitro* hardening of plantlets. For this purpose, mycelium was harvested by simply draining the culture medium from broth cultures leaving the fungus in the flask which was washed several times with double sterile distilled water sieving the fungus through double-lined cheese cloth each time. Mycelium (about 50 mg fresh weight per plant) was mixed with autocalved Soilrite™. Prior to mixing, the mycelium was mechanically homogenized in sterile distilled water. The Soilrite™-fungus (SF) mix was distributed (150 g) in culture bottles (400 ml) and in each bottle 3 rooted shoots, derived from multiplying cultures as described in preceding sections, were implanted. The mycelium suspension – prepared by homogenizing mycelium in water (1:1 w/v) – was also applied to the roots by dipping them in the mycelial suspension prepared above for about 5 seconds prior to transferring the plants to SF. In this way, the roots were coated with a thin layer of mycelial suspension. It provided inoculum both on the root surface and in Soilrite™ sufficient for symbiosis to occur. In all, 75 plantlets (derived from un-inoculated culture) were inoculated. Non-inoculated plantlets (75) of similar size were also maintained on plain Soilrite™ as a control. Hardening and acclimatization of plantlets was achieved using the procedure described by Tak (1993). Initially Soilrite™ was moistened with 1/4 MS salts solution and subsequently watered whenever required. Bottles were kept under standard greenhouse conditions (25-28°C, 55-80% relative humidity and 400 µmol $m^{-2}s^{-1}$ light intensity under natural daylight conditions) and their caps were gradually (over three weeks) opened. Thirty-days-old plantlets were transplanted into polybags containing soil, Soilrite™ and farmyard manure (a traditional decomposed mixture of cattle dung, urine, straw, litter and residues from the fodder fed to cattle providing around 0.4 to 1.5% N, 0.3-0.9% P_2O_5 and 0.3-1.9% K_2O to plants; http://www.indiaagronet.com/indiaagronet/Manuers_fertilizers/contents/farm_yard_manure.htm) in a 1:1:1 ratio. At the time of transplantation, root segments from treated and untreated samples were harvested from randomly selected individuals (five each from treated and untreated controls) to examine the colonization by *P. indica*. These plants, after a week's growth in the greenhouse were subsequently transferred to a nursery shed for acclimatization where 70% shade, provided by green Agro-shade Net (Insta Shade, Vadodara, India), allowed plants to grow in open natural environment while remaining protected from direct sunlight.

Staining of root samples and microscopic examination

The root segments harvested from 30-day-old plantlets during transplantation were washed thoroughly in running tap water, cut into 1.0 cm pieces and treated overnight with 10% KOH solution at room temperature. Thereafter, the roots were washed 3-5 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 plant growth and root colonization

The growth performance was assessed in 3-month-old plants. The fresh and dry weight (shade air-dried), shoot and root length, internode number and length, stem diameter, number of leaflets and average leaf area, survival percentage, and percentage root colonization of the inoculated and non-inoculated plants were recorded. For observations and data collection, 10 plant samples from each treatment were collected randomly. The harvested plants were washed gently under running water keeping the roots intact. To determine the endophyte dependency (ED) of *F. limonia*, the formula given by Gerdemann (1975) was used:

$$ED = \frac{\text{Dry weight of inoculated plants}}{\text{Dry weight of uninoculated plants}} \times 100$$

For assessment of root colonization, the method proposed by Giovannetti and Mosse (1980) was followed. The root segments (1.0 cm) were selected at random from the stained samples and observed under an Olympus KH 29035 binocular microscope (X400). In all, 25 segments were examined under the microscope and the percentage colonization was calculated as follows:

$$\text{Percentage colonization} = \frac{\text{Number of root segments colonized}}{\text{Total number of segments examined}} \times 100$$

Biochemical profiling of colonized plants

The *P. indica*-inoculated and non-inoculated plantlets were also assessed and compared for total proteins (Bradford 1976), proline (Bates *et al*. 1973) and enzymes, namely peroxidase (Anonymous 1973), nitrate reductase (Mahadevan and Sridhar 1986) and superoxide dismutase (Beauchamp and Fridovich 1971).

Statistical analysis

All experiments were conducted in triplicate in CRD. Means and standard deviations were calculated and the Student's *t*-test was applied to evaluate the significance of differences at $p \leq 0.05$, wherever required (Bailey 1995).

RESULTS

In vitro-derived shoots of *F. limonia* developed single, thick and firm roots on SRM (inoculated without *P. indica* treatment) within 15 days of inoculation (**Fig. 2A**). These roots grew normally in SRM, and when transferred to Soilrite^{TM}, helped the successful establishment of plantlets thereafter. A rooting response of nearly 60% was achieved through this method (**Table 1**). Attempts to achieve simultaneous *in vitro* root induction and colonization with *P. indica* were also made. However, treatment with *P. indica at* the basal cut end of *in vitro*-derived shoots of *F. limonia*, prior to inoculation on SRM, suppressed root induction (**Fig. 2B**). Only 30% of plants produced roots compared to 60% in non-inoculated controls. The roots were small and few (**Table 1**).

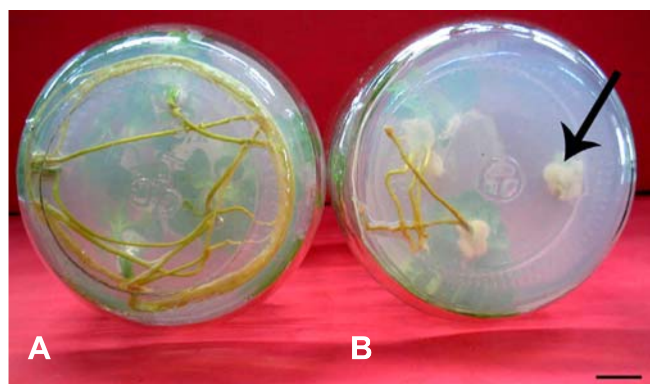

Fig. 2 Suppression of root induction (indicated by arrow) on inoculation with *P. indica* in *F. limonia*. (**A**) Control; (**B**) shoots inoculated with *P. indica*. Bar = 1.0 cm.

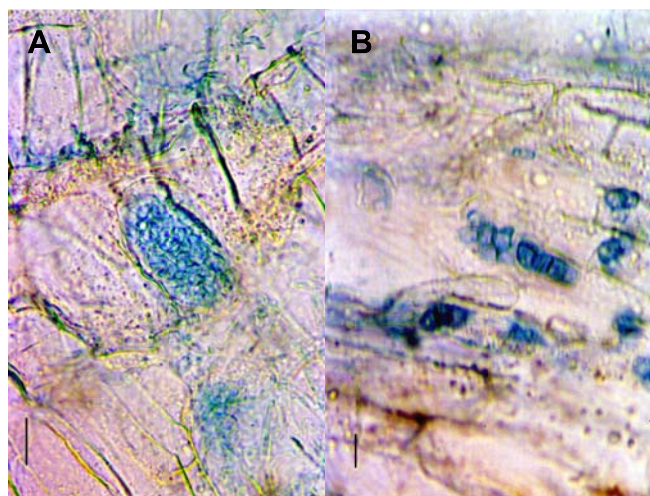

Fig. 3 Roots of *F. limonia* colonized with P. *indica*. (**A**) Coils and (**B**) round bodies in cortical cells. Bar = 10.0 µm.

Table 1 Effect of *Piriformospora indica* on *in vitro* root induction in *Feronia limonia*. Mean values ± SD of the 10 samples are presented except for the rooting response which was calculated on 25 plants each for the given treatments.

Treatment	Non-inoculated	Inoculated
Rooting response (%)	60	30
Root number	2.9 ± 0.56 a	0.8 ± 0.78 b
Root length (cm)	6.0 ± 0.64 a	4.5 ± 0.43 b

Figures in the same row followed by different letters differ significantly at $p \leq 0.05$ according to Student's *t*-test.

Table 2 Effect of root colonization by *Piriformospora indica* on growth and development in tissue culture plantlets of *Feronia limonia* during hardening and acclimatization. Mean values ± SD of the 10 samples are presented.

Growth Parameters	Non-inoculated	Inoculated
Shoot height (cm)	11.5 ± 1.35b	15.9 ± 1.19a
Root length (cm)	16.2 ± 2.39 b	22.4 ± 1.91a
Number of internodes	8.0 ± 1.10 b	11.4 ± 1.17a
Internode length (cm)	1.2 ± 0.13 b	1.8 ± 0.12 a
Stem diameter (mm)	1.7 ± 0.60 b	2.9 ± 0.30 a
Number of leaflets	16.0 ± 2.70 b	25.0 ± 3.30 a
Leaf area (mm^2)	26.7 ± 4.16 b	39.8 ± 4.26 a
Fresh weight (g)	0.9 ± 0.14 b	1.1 ± 0.12 a
Dry weight (g)	0.3 ± 0.02 b	0.6 ± 0.05a
Per cent root colonization	-	85%
Endophyte dependency	-	193%

Figures in the same row followed by different letters differ significantly at $p \leq 0.05$ according to Student's *t*-test.

Table 3 Influence of *Piriformospora indica* on survival percentage of tissue culture plantlets of *Feronia limonia* during *in vitro* hardening and subsequent growth in polybags under nursery shed conditions. Mean values ± SD of the 6 samples are presented.

Treatment	Survival percentage after 30 d of *in vitro* hardening on Soilrite^{TM} in greenhouse (n=75)	Survival percentage after 3 months of hardening and acclimatization in potting mix under nursery shed (n=75)
Non-inoculated (%)	75	80
Inoculated (%)	98	92

Due to the negative effect of this fungus on root induction, shoots were first allowed to root on SRM and then inoculated with *P. indica*. *In vitro* plants of *F. limonia*, when cultivated with *P. indica*, showed successful root colonization. The hyphae of the fungus coming in contact with the roots penetrated the root system intercellularly. Colonization was observed intracellularly in epidermal and cortical cells where it formed coils and branches or round bodies (**Figs. 3A, 3B**). *P. indica* did not invade the stellar tissues or traverse upwards into the shoot. The percentage root colonization observed after 30 days of inoculation with *P. indica* was 85% (**Table 2**). Compared to the treated plants, roots of control plants contained no fungal structures. MD is used as an index to compare receptivity of different plant species to AM fungi. This can also be used for other endophytes such as *P. indica*. In the present study the term ED is used instead of MD as the test organism does not develop a typical mycorrhizal association. An ED value of 193% for *F. limonia* was observed (**Table 2**).

Four weeks after the transfer of untreated plantlets to Soilrite^{TM} and gradual exposure to the *ex vitro* environment in the greenhouse, nearly 75% survival was observed (**Table 3**). After transplantation in the potting mix and 3 months of growth under nursery shed conditions, 80% survival was

Fig. 4 Growth performance of tissue cultured *F. limonia* plantlets under nursery shed conditions. (**A**) Control; (**B**) inoculated with *P. indica*. Bar = 5.0 cm.

Table 4 Biochemical parameters of tissue-culture raised plantlets of *Feronia limonia* inoculated with *Piriformospora indica* during *in vitro* hardening.

Biochemical parameters	Non-inoculated	Inoculated
Proteins (mg/g fresh weight of tissues)	21.4 ± 1.35 b	22.5 ± 1.04 a
Proline (mg/g fresh weight of tissues)	7.0 ± 0.53 b	11.0 ± 1.26 a
Peroxidases (Δ/min/g fresh weight of tissues)	6000 ± 500 b	7800 ± 300 a
SOD (U/g fresh weight of tissues)	980 ± 114 b	1200 ± 101 a
Nitrate reductase (mM of nitrite formed per 20 min/g fresh weight of tissues)	33.6 ± 1.63 b	37.2 ± 2.22 a

Figures in the same row followed by different letters differ significantly at $p \leq 0.05$ according to Student's *t*-test.

observed (**Table 3**). An average above-ground height of 11.5 cm was attained by control plants (**Table 2**). The shoots developed nearly 8 internodes with an average diameter of 2 mm. The average total of leaflets produced per plant was 16, with an area of about 27 mm^2 per leaflet. The roots attained a length of 16 cm. The total fresh and dry biomass per plant was recorded to be 0.9 and 0.3 g, respectively after 3 months' growth (**Table 2**).

In comparison to the untreated controls, the plants inoculated with *P. indica* showed 98% survival (**Table 3**) after 4 weeks' inoculation. When transplanted to the potting mix, the treated plants showed vigorous and enhanced growth (**Fig. 4**) and only 8% mortality was recorded after 3 months' growth (**Table 3**). An average shoot height of 16 cm with about 12 internodes having an average girth of 3.0 mm was recorded for these plants. Each plant had about 26 leaflets each with an area of 40 mm^2 (**Table 2**). The fresh and dry weights were about twice those of the control (1.12 g and 0.58 g, respectively).

Total proteins and proline contents and peroxidase, nitrate reductase and superoxide dismutase enzyme activities recorded for plants biotized with *P. indica* invariably showed higher values than non-inoculated control (**Table 4**).

DISCUSSION

A positive influence of root colonization with *P. indica* on vegetative growth and development in micropropagated plants of *F. limonia* was observed in this study. The treated plants showed pronounced growth relative to the non-inoculated control. Similar reports of increased growth on inoculation with *P. indica* have been observed for *Spilanthes calva* and *Withania somnifera* (Rai *et al*. 2001). Varma *et al.* (1999) reported an increase in plant height, fresh and dry biomass and larger leaf area in micropropagated *Artemisia annua*, *Bacopa monnieri* and tobacco. The differences in growth observed between treated and control plants were suggested to be caused by greater absorption of water and nutrients due to extensive colonization of roots by *P. indica* (Rai *et al*. 2001). Such plants show enhanced P uptake resulting in an increase in fresh and dry weights (Sudha *et al.* 1998). The more intense root proliferation in treated plants here may be due to the synthesis of yet unidentified extracellular phytohormones by *P. indica* (Singh *et al*. 2000; Varma *et al*. 2001).

Early root differentiation in tissue culture-raised plantlets has been reported by Sahay (1999) on inoculation with *P. indica*. In contrast, during the present study, the induction of roots on *in vitro*-derived shoots of *F. limonia* was completely suppressed when inoculated with *P. indica*. A similar inhibition in rooting performance was observed in micropropagated plums by Fortuna *et al.* (1998), using AM fungus. Hetrick *et al.* (1988) argued that this root inhibition might be caused by immobilization of nutrients by AM or competition for available nutrients. In our case also, a similar behaviour by the endophyte might have caused suppressed root growth. Varma *et al.* (1999) claimed that for biohardening of tissue culture plants, inoculation must be involved after roots are formed.

Changes in biochemical performance were also recorded in *P. indica*-treated and untreated plants. Enzymes like peroxidase, superoxide dimutase and nitrate reductase are generally induced during stress (Caravaca *et al*. 2005). In the present study, the increased peroxidase, superoxide dismutase and nitrate reductase activity in biotized or bioprimed plantlets indicated a better defense mechanism in the tissue and high oxidation rates which supported plantlet development. Peroxidase activity has been shown to follow a specific pattern throughout the rooting process, where it can be used as marker and predictor of rooting performance (Gaspar *et al*. 1992). In the present case also, the increased peroxidase activity among biotized plants was coincident with a better root system. The increase in values of nitrate reductase activity and total protein contents indicated very high state of cell N_2 metabolism and protein synthesis.

Inoculation of plantlets with microbial symbionts has been recommended for reducing the stress of acclimatization, providing faster growth and better establishment of micropropagated plants. *P. indica* promises to be an excellent candidate for biological hardening of micropropagated plantlets as the fungus rendered more than 90% survival rate of the transferred plantlets of *Nicotiana tabacum* L. and *Bacopa monnieri* L. Wett. (Varma *et al.* 1999) and *Terminalia bellerica* (Suthar and Purohit 2008). In this study also, the tissue culture raised plants of *F. limonia* showed better survival percentage (90%) than the control (60%) during hardening and acclimatization. The present investigation highlights the role of *P. indica* as a potential biohardening agent for difficult-to-harden plants.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. Ajit Varma, Jawaharlal Nehru University, New Delhi (now at Amity University, Noida, (UP, India) for kindly providing cultures of *P. indica*. The financial support provided by Council of Scientific and Industrial Research (CSIR), New Delhi, India to one of the authors (S Vyas) is gratefully acknowledged.

REFERENCES

Anonymous (1973) Enzymes, enzyme reagents, related biochemicals. In: *Worthington Enzyme Manual*, Worthington Biochemical Corporation, Freehold, New Jersey, 216 pp

Bailey NTJ (1995) *Statistical Methods in Biology*, Cambridge University Press, Cambridge, UK, 276 pp

Balla I, Vértesy J, Köves Pechy K (1997) Acclimation results of micropropagated black locust (*Robinia pseudoacacia* L.) improved by use of microorganisms. In: Cassells AL (Ed) *Pathogen and Microbial Contamination Management in Micropropagation*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 351-354

Bates LS, Waldren RP, Jeare ID (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205-207

Beauchamp CO, Fridovich I (1971) Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276-287

Bradford MM (1976) A rapid and sensitive method for the quantitation of

microgram quantity of protein utilizing the principle of protein dye binding. *Annals of Biochemistry* **72**, 248-254

Caravaca F, Alguacil MM, Hernández JA, Roldán A (2005) Involvement of antioxidant enzyme and nitrate reductase activities during water stress and recovery of mycorrhizal *Myrtus communis* and *Phillyrea angustifolia* plants *Plant Science* **169**, 191-197

Dave N, Purohit SD (2004) *In vitro* growth and shoot multiplication of *Achras sapota* in a controlled carbon dioxide environment. *Biologia Plantarum* **48**, 621-624

Desjardins Y (1995) Overview of factors influencing photosynthesis of micropropagated plantlets and their effect on acclimatization. In: Carre F, Chagvardieff P (Eds) *Ecophysiology and Photosynthetic in Vitro Cultures*, CEA, Aix-en-Provence, France, pp 145-160

Desjardins YF, Laforge C, Lussier C, Gosselin A (1988) Effect of CO_2 enrichment and high photosynthetic photon flux on the development of autotrophy and growth of tissue cultured strawberry, raspberry and asparagus plants. *Acta Horticulturae* **230** 45-53

Donnelly DJ, Vidaver WE, Lee KY (1985) The anatomy of tissue cultured red raspberry prior to and after transfer to soil. *Plant Cell, Tissue and Organ Culture* **4**, 43-50

Elmeskaoui A, Damont JJP, Poulin MJ, Piché Y, Desjardins Y (1995) A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets *in vitro*. *Mycorrhiza* **5**, 313-319

Fortuna P, Morini S, Giovannetti M (1998) Effects of arbuscular mycorrhizal fungi on *in vivo* root initiation and development of micropropagated plum shoots. *Journal of Horticultural Science and Biotechnology* **73**, 19-28

Gaspar T, Kevers C, Hausman JF, Berthon JY, Ripetti V (1992) Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. *Agronomie* **12**, 757

Gerdemann JW (1975) Vesicular-arbuscular-mycorrhizae. In: Torrey JG, Clarkson DT (Eds) *The Development and Function of Roots*, Academic Press, London, pp 575-591

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular – arbuscular mycorrihizal infection in roots. *New Phytologist* **84**, 489-500

Herman EB (1996) Beneficial effects of bacteria and fungi on plant tissue cultures. *Agricell Reports* **27**, 26-27

Hetrick BAD, Leslie JF, Thompson-Wilson G, Gerschefske-Kitt D (1988) Physiological and topological assessment of effects of a vesicular-arbuscular mycorrhizal fungus on root architecture of big bluestem. *New Phytologist* **110**, 85-96

Hiregoudar LV, Murthy HN, Hema BP, Hahn EJ, Paek KY (2003) Multiple shoot induction and plantlet regeneration of *Feronia limonia* (L.) Swingle. *Scientia Horticulturae* **98**, 357-364

Hooker JE, Gianinazzi S, Vestberg M (1994) The application of arbuscular mycorrhizal fungi to micropropagation systems: an opportunity to reduce chemical inputs. *Agricultural Science Finland* **3**, 227-232

Hossain M, Biswas BK, Karim MR, Rahaman S, Islam R, Joarder OI (1994) *In vitro* organogenesis of elephant apple (*Feronia limonia*). *Plant Cell, Tissue and Organ Culture* **39**, 265-268

Kaefer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. *Advances in Genetics* **19**, 33-131

Kirtikar KR, Basu BD (1993) *Indian Medicinal Plants* (Vol I), Orient Enterprises, Dehradun, India, pp 496-498

Mahadevan A, Sridhar R (1986) Methods *in Physiological Plant Pathology* (3rd Edn), Sivakami Publications, Madras, India, pp 88-91

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Nowak J (1998) Benefits of *in vitro* "biotization" of plant tissue cultures with microbial inoculants. *In Vitro Cellular and Developmental Biology – Plant* **34**, 122-130

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**, 158-161

Preece JE, Sutter EG (1991) Acclimation of micropropagated plants to the greenhouse and field. In: Debergh PC, Zimmerman RH (Eds) *Micropropagation, Technology and Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 71-93

Purohit SD, Tak K (1992) *In vitro* propagation of an adult tree *Feronia limonia* L. through axillary branching. *Indian Journal of Experimental Biology* **30**, 377-379

Rahaman MM, Gray AI (2002) Antimicrobial constituents from stem bark of *Feronia limonia*. *Phytochemistry* **59**, 73-77

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. *Mycorrhiza* **11**, 123-128

Sahay NS (1999) Interaction of *Piriformospora indica* with tissue culture raised plants. PhD thesis. Jawaharlal Nehru University, New Delhi

Saima Y, Das AK, Sarkar KK, Sen AK, Sur B (2000) An antitumor pectic polysaccharide from *Feronia limonia*. *Biological Macromolecules* **27**, 333-335

Singh A, Sharma J, Rexer K-H, Varma A (2000) Plant productivity determinants beyond minerals, water and light. *Piriformospora indica*: a revolutionary plant growth promoting fungus. *Current Science* **79**, 101-106

Sudha, Hurek T, Varma A (1998) Active translocation of phosphate (P^{32}) to rice and carrot by *Piriformospora indica*. In: Ahonen Jonnarth U, Danell E, Fransson P, Karen O, Lindahl B, Rangel I, Finlay R (Eds) *Second International Congress on Mycorrhiza*, July 5-10, Uppsala, Sweden

Suthar R, Purohit SD (2008) Root colonization and improved growth performance of micropropagated *Terminalia bellerica* Roxb. plantlets inoculated with *Piriformospora indica* during *ex vitro* acclimatization. *International Journal of Plant Developmental Biology* **2**, 133-136

Tak K (1993) Tissue culture studies on some forest tree species of Aravallis in South Rajasthan. PhD thesis, Mohanlal Sukhadia University, Udaipur, India, 113 pp

van Huylenbroeck JM, Debergh PC (1996) Physiological aspects in acclimatization of micropropagated plantlets. *Plant Tissue Culture and Biotechnology* **2**, 136-141

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer K–H, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: An axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock X (Ed) *The Mycota IX Fungal Associations*, Springer-Verlag, Heidelberg, pp 125-150

Varma A, Verma S, Sudha, Sahay N, Buetehorn B, Franken P (1999) *Piriformospora indica* – a cultivable plant growth–promoting root endophyte with similarities to arbuscular mycorrhizal fungi. *Applied and Environmental Microbiology* **65**, 2741-2744

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Buetehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. Nov., a new root-colonizing fungus. *Mycologia* **90**, 896-903

Vosátka M, Albrechtová J (2008) Theoretical aspects and practical uses of mycorrhizal technology in floriculture and horticulture. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol V), Global Science Books, Isleworth, UK, pp 466-479

Vyas S, Joshi N, Tak K, Purohit SD (2005) *In vitro* adventitious shoot bud differentiation and plantlet regeneration in *Feronia limonia* L. (Swingle). *In Vitro Cellular and Developmental Biology – Plant* **7**, 296-302

Vyas S, Purohit SD (2003) *In vitro* growth and shoot multiplication of *Wrightia tomentosa* (Roxb.) Roem et Schult in a controlled carbon dioxide environment. *Plant Cell, Tissue and Organ Culture* **75**, 283-286

Wilhelm E, Arthofer W, Schafleitner R (1997) *Bacillus subtilis* an endophyte of chestnut (*Castanea sativa*) as antagonist against chestnut blight (*Gyphonectaria parasitica*). In: Cassells A (Ed) *Pathogen and Microbial Contamination, Management in Micropropagation*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 331-337

Ziv M (1991) Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh PC, Zimmerman RH (Eds) *Micropropagation Technology and Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 45-69