Mycorrhiza (2001) 11:123–128
DOI 10.1007/s005720100115

ORIGINAL PAPER

Mahendra Rai · Deepak Acharya · Archana Singh
Ajit Varma

Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial

Accepted: 14 May 2001 / Published online: 10 July 2001

Abstract The medicinal plants *Spilanthes calva* and *Withania somnifera* were inoculated with *Piriformospora indica*, a plant growth-promoting root endophyte, in nurseries and subsequently transferred to the field. A significant increase in growth and yield of both plant species was recorded relative to uninoculated controls. Shoot and root length, biomass, basal stem, leaf area, overall size, number of inflorescences and flowers and seed production were all enhanced in the presence of the fungus. Net primary productivity was also higher than in control plants. The results clearly indicate the commercial potential of *P. indica* for large-scale cultivation of *S. calva* and *W. somnifera*.

Keywords Medicinal plants · *Spilanthes calva* · *Withania somnifera* · *Piriformospora indica* · Field trials

Introduction

The potential of arbuscular mycorrhizal (AM) fungi for growth promotion of plants has been well established (Azcon-Aguilar et al. 1994; Bagyaraj and Varma 1995; Fortuna et al. 1992; Gianinazzi et al. 1990; Morte et al. 1996; Varma 1995, 1998, 1999; Varma and Schuepp 1995). However, there are only few reports of the enhancement of growth by in vitro culturable endophytes (Dix and Webster 1995; Schulthess and Faeth 1998). *Piriformospora indica* has been characterized as a plant growth-promoting, root-colonizing fungus (Blechert et al. 1999; Franken et al. 1998; Singh et al. 2000; Varma et al. 1999; Verma et al. 1998). This axenically culturable fungus resembles AM fungi in several functional and physiological characteristics (Singh et al. 2000; Varma et al. 1999, 2001). It improves the growth and biomass of a wide host range and is an efficient phosphate solubilizer and transporter (Sudha et al. 1998; Varma et al. 2001). Like AM fungi, *P. indica* does not colonize the roots of members of Crucifereae or the Myc- mutants of soybean (*Glycine max*) and pea (*Pisum sativum*) (Singh 2001). More than 90% of the micropropagated plantlets of tested hosts treated with the fungus survive on transfer from laboratory to open field conditions (Sahay and Varma 1999, 2000) due to callus differentiation and excessive root proliferation. The fungus also protects the plantlets from some potent root pathogens (Varma et al. 2001).

Spilanthes calva DC and *Withania somnifera* Dunal are important tropical medicinal plants commonly used in ayurveda (Indian natural therapy) and other traditional systems of medicine (Bhargava and Singh 1978; Dey 1980; Sahni and Srivastava 1993; Sankaran 1984; Tripathi et al. 1996). *W. somnifera* is also known as Indian Ginseng and belongs to the family Solanaceae. More than 91 pharmaceutical products are produced from the roots of this plant. *S. calva*, a member of Asteraceae, is also a medicinal herb, known as "toothache plant" or "virus blocker", and is well-known for enhancing immunity. Because of their high medicinal value, there is an increasing demand for these plants in national and international markets. Thus, enhancement of the growth and bioactive agents of these plants is desirable. This may be achieved by inoculation of the roots with microorganisms like AM fungi or other growth-promoting endophytes.

The main objective of the present study was to evaluate the influence of *P. indica* on the growth and morphology of *S. calva* and *W. somnifera* after transplanting from nursery to field conditions.

M. Rai · D. Acharya
Danielson College, P.O. Box 41, Chhindwara, M.P., India

A. Singh · A. Varma (✉)
School of Life Sciences, Jawaharlal Nehru University,
New Delhi, 110 067, India
e-mail: ajitkrvarma@hotmail.com
Fax: +91-11-6165886

Materials and methods

Germplasm of medicinal plants

Seeds of *S. calva* and *W. somnifera* were provided by Shrinath Agrawal, a local medicinal plant grower of Chhindwara, Madhya Pradesh, Central India.

Inoculum

The fungus was maintained on Kaefer medium (Kaefer 1977). Broth cultures were inoculated with agar fungal discs and incubated at 30±2°C under constant shaking conditions (100 rpm) in the dark for 10 days. Fungal mycelium was harvested and washed several times with sterile water. About 10 g surface-sterilized (2% sodium hypochlorite for 10 min; Verma et al. 1998) seeds of *S. calva* and *W. somnifera* were mixed with a mechanically homogenized culture of *P. indica* in sterile water. The inoculum was applied at 1% w/w of seed. The technique of inoculation was as described earlier (Varma et al. 1999).

Nursery bed preparation

Eight nursery beds (5×1 m^2) were prepared at Choubitker Farm, Chandangaon, Chhindwara, Central India. The nursery bed soil was prepared by adding a mixture of soil: sand: farmyard manure (3:1:1). The soil mixture was disinfected with 1% formalin for 48 h and left for 5 days to aerate to eliminate excess formalin.

Seed sowing and plant maintenance

Piriformospora indica-treated and untreated seeds were sown in furrows in the nursery beds. The seeds of *S. calva* were shallow sown as they generally do not germinate or only slowly when deep sown. In the case of *W. somnifera*, a 0.5-cm layer of soil was added after broadcasting the seeds. Two-week-old seedlings were transferred to a nearby non-disinfected field with brown sand-loam soil, pH 7.3, with N, P and K at 38.2, 1.8 and 51.5 g/m^2, respectively. *P. indica* at a density of 50 mg/ml mycelia (fresh weight) was reinoculated along the juvenile roots, thus providing inoculum to the plants at two stages: at the time of sowing (in nursery beds) and at seedling transfer to the field trial site. Control seeds and seedlings were treated with an amount of autoclaved mycelium equal to that used for inoculation. The plants were irrigated on alternate days and weeds were hand eradicated periodically.

Data and sample collection

The shoot length, stem diameter, leaf area, flower dimensions, number of seeds per inflorescence/per fruit and percent root colonization were recorded at plant maturity (after 90 days). The fresh and dry weights of surface and underground parts were also measured. Ten plant samples from each treatment were collected randomly and shade-air dried. The harvested plant material was washed gently with water in a wire-cage to avoid breaking roots. The net primary productivity was calculated on a dry weight basis and expressed as g/plant/day.

The basal area of plant stem was determined by the formula suggested by Misra (1968):

$$\text{Average basal area / Sq cm} = \frac{\pi r^2(\text{CS1})}{2} \quad (1)$$

Endophyte dependency

Endophyte dependency of *S. calva* and *W. somnifera* was determined using the formula given by Gerdemann (1975):

$$\text{ED} = \frac{\text{Dry weight of inoculated plants}}{\text{Dry weight of uninoculated plants}} \times 100 \quad (2)$$

ED is used instead of MD to designate endophyte dependency.

Staining of root samples

Roots of *S. calva* and *W. somnifera* were washed thoroughly in running tap water, cut into 1-cm pieces and treated overnight with 10% KOH solution at room temperature. Thereafter, the root pieces 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 (×400).

Assessment of root colonization

The method proposed by Giovannetti and Mosse (1980) was followed for assessment of root colonization. The root-pieces (1 cm) were selected at random from the stained samples and mounted on grid-intersect glass slides. In all, 100 root pieces per sample were observed and percent infection was calculated as follows:

$$\text{Percent colonization} = \frac{\text{No. of root segments colonized}}{\text{Total no. of segments observed}} \times 100 \quad (3)$$

Statistical analysis

Means and standard deviations were calculated and a *t*-test applied to evaluate the significance of differences (Bailey 1995).

Results and discussion

A pronounced growth response following inoculation with *P. indica* was observed in both *S. calva* and *W. somnifera* (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12). The plants treated with *P. indica* were superior in development to control plants (Tables 1, 2, 3). A significant increase in shoot length was observed in inoculated plants. Microscopic examination of stained root samples revealed a high colonization by *P. indica* of 62 and 73% root length in *S. calva* and *W. somnifera*, respectively.

The basal stem and leaf areas of treated plants were also enhanced (Table 2). Interestingly, some large, 2.3 (±0.06) × 1.54 (±0.38) cm^2, kidney-shaped inflorescences were observed on inoculated *S. calva* plants together with the normal, round inflorescences (Fig. 5). These kidney-shaped inflorescences were never observed in control plants (Fig. 6). The lengths of the inflorescences and the number of flowers on inoculated *S. calva* plants were also increased relative to controls (Table 2; Fig. 7, 8). Similarly, the number of flowers on the inoculated plants of *W. somnifera* was higher (Table 2) than on controls. For both medicinal plant species, seed counts were higher for treated than for control plants.

The overall root biomass of the inoculated plants was higher than that of the corresponding controls (Figs. 9, 10, 11, 12). The fresh and dry weights of both underground and above-ground parts of *S. calva* and *W. somnifera* inoculated plants were higher than the controls (Table 3). The net primary productivity of inoculated *S.*

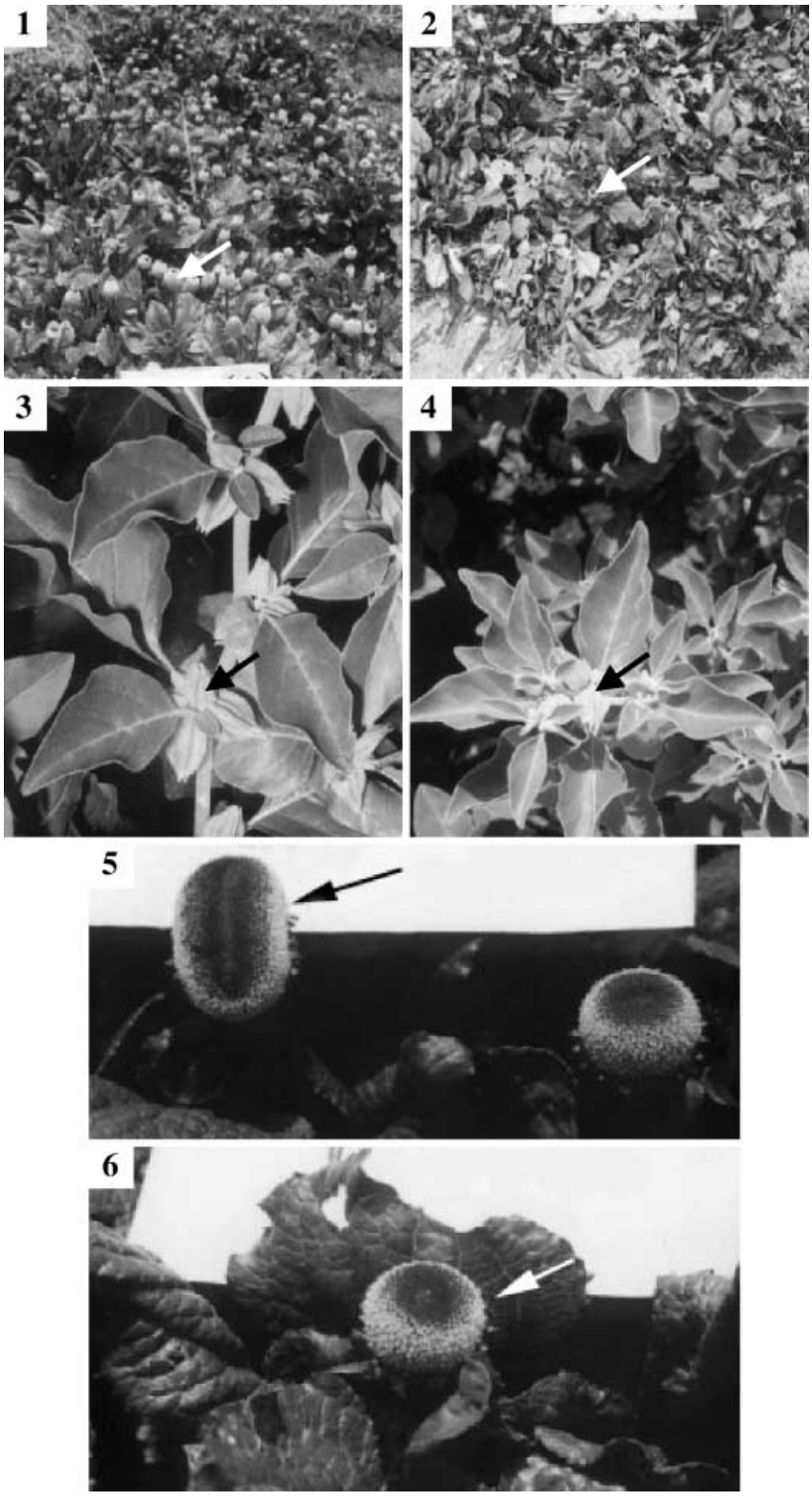

Fig. 1 Growth of *Spilanthes calva* and *Withania somnifera* inoculated with *Piriformospora indica*. Surface-sterilized seeds were sown in furrows in nursery beds. *P. indica* was inoculated at the rate of 1% w/w of seeds at the time of seed sowing and later on transfer of the seedlings to the field. Inclusion of autoclaved fungal mycelium in equal quantity served as controls. *Arrow* shows an increase in size and abundance of inflorescences in inoculated *S. calva*

Fig. 2 As Fig. 1. *Arrow* shows the small-sized, infrequently occurring inflorescences in uninoculated controls of *S. calva*

Fig. 3 As Fig. 1, showing the vigorous growth of foliage and inflorescences (*arrow*) in inoculated *W. somnifera*

Fig. 4 As Fig. 1, showing the poor growth of foliage with small inflorescences (*arrow*) in uninoculated controls of *W. somnifera*

Fig. 5 As Fig. 1. *Arrow* indicates a large kidney-shaped inflorescence frequently observed in addition to normal heads in *S. calva* inoculated with *P. indica*

Fig. 6 As Fig. 1. *Arrow* shows normal round inflorescence heads in an uninoculated control of *S. calva*

calva and *W. somnifera* plants was 0.06 and 0.23 g/plant/day, respectively. These values were higher than those of control plants (0.02 and 0.12 g/plant/day, respectively).

MD is used as an index to compare receptivity of different plant species to AM fungi (Gerdemann 1975). 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. The ED value was 211.13 for *S. calva* and 671.90 for *W. somnifera*. These data suggest that *P. indica* has a greater influence on the growth of *W. somnifera* than on that of *S. calva*.

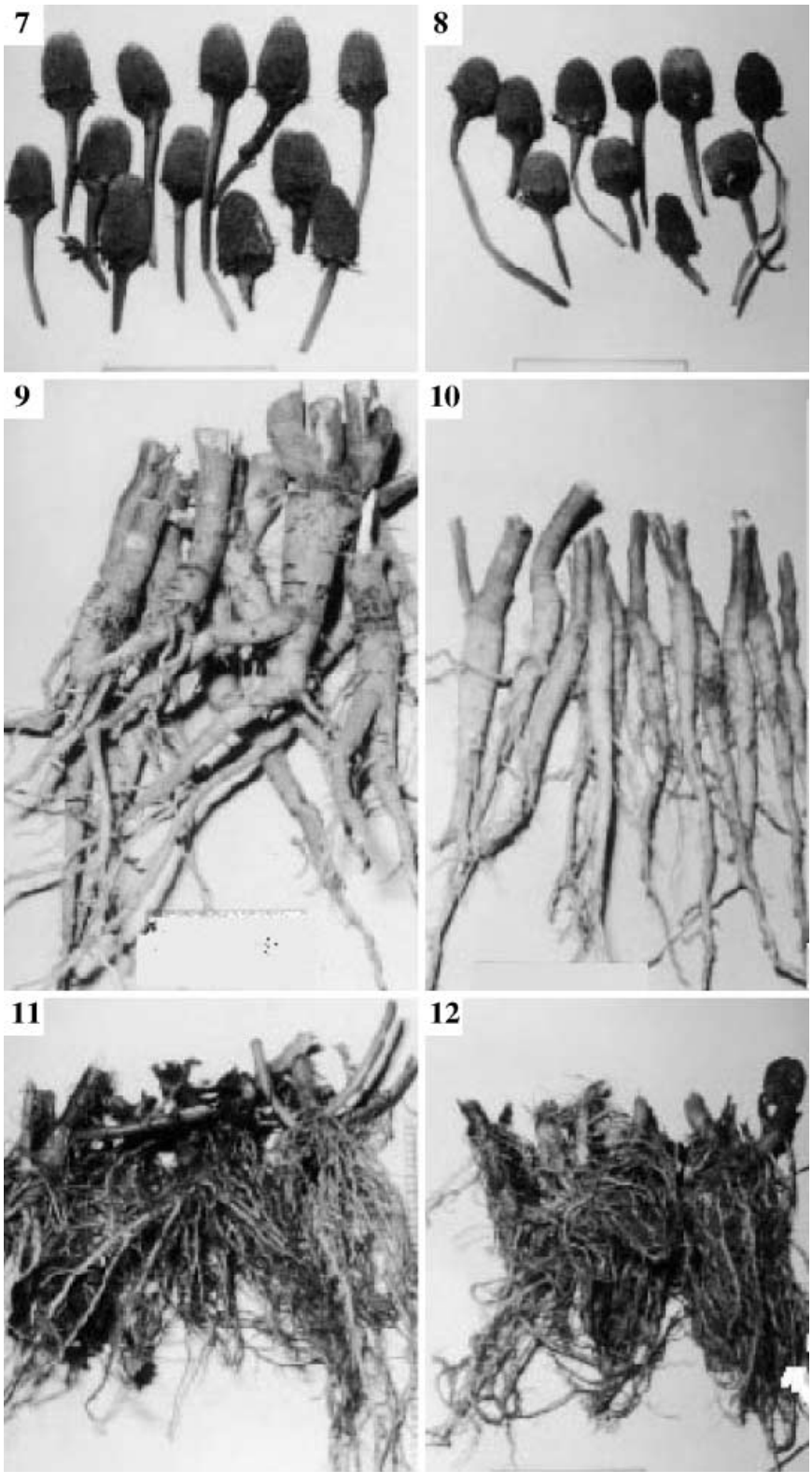

Fig. 7 Influence of *P. indica* on the development of *S. calva* and *W. somnifera*: increase in the number and size of inflorescences of *S. calva* inoculated with *P. indica*, harvested at plant maturity

Fig. 8 As Fig. 7. Inflorescence of an uninoculated control of *S. calva* harvested at plant maturity

Fig. 9 As Fig. 7, showing the prominent increase in biomass and proliferation of roots of *S. calva* inoculated with *P. indica*

Fig. 10 As Fig. 7, showing the root biomass of an *S. calva* uninoculated control

Fig. 11 As Fig. 7, showing the prominent increase in biomass and proliferation of roots of *W. somnifera* inoculated with *P. indica*

Fig. 12 As Fig. 7, showing the root biomass of an *W. somnifera* uninoculated control

The MD of a host plant can be altered by factors such as soil-type, soil P content, mycorrhizal species (Azcon and Ocampo 1981; Menge et al. 1978). Amongst the reasons proposed for differences in MD in different plants or varieties of the same species, Baylis (1975) reported that root-hair length and root thickness can determine the MD level. Rajapakse and Miller (1988) observed that the average length of fine roots was negatively correlated with MD in cowpea. The more intense root proliferation in treated plants observed here may be due to the synthe-

Table 1 Influence of *Piriformospora indica* on shoot and root length and on percent root colonization of *Spilanthes calva* (*S.c.*) and *Withania somnifera* (*W.s.*) in a field trial. The control plants were treated with an equal amount of autoclaved mycelium. All values are means ± S.D. Mean values are significantly different at $P<0.05$

Treatment	Shoot length (cm)		Root length (cm)		Percent root colonization	
	S.c.	*W.s.*	*S.c.*	*W.s.*	*S.c.*	*W.s.*
+ *Piriformospora indica*	28.76 (±0.68)	67.3 (±0.26)	4.46 (±0.45)	16.53 (±0.25)	62.0 (±2.08)	73.0 (±2.64)
Control	25.16 (±0.20)	42.23 (±0.2)	2.13 (±0.15)	13.03 (±0.15)	0	0

Table 2 Influence of *Piriformospora indica* (*P.i.*) on morphology and growth of host plants, 90 days after inoculation in a field trial. The control plants were treated with an equal amount of autoclaved mycelium. All values are means ± S.D. Mean values are significantly different at $P<0.05$. Abbreviations as in Table 1

Hosts	Treatment	Basal stem area cm^2	Leaf area cm^2	Diameter of inflorescence cm	Length of inflorescence cm	No. of flowers or inflorescences	No. of seeds or fruits
S.c.	+ *P.i.*	7.06 (±0.47)	37.67 (±2.28)	5.06 (±0.32)	2.49 (±0.09)	48.57 (±0.4)	1006 (±7.63)
	Control	4.11 (±0.57)	26.00 (±2.87)	4.16 (±0.76)	1.48 (±0.03)	11.50 (±3.6)	716 (±0.36)
W.s.	+ *P.i.*	11.40 (±0.61)	45.59 (±0.34)	–	–	307.40 (±0.53)	46.33 (±5.77)
	Control	5.57 (±0.49)	13.08 (±0.73)	–	–	81.80 (±1.57)	35.33 (±4.93)

Table 3 Influence of *P. indica* on the biomass of field-grown *S. calva* and *W. somnifera* plants. The control plants were treated with equal amounts of autoclaved mycelium. Abbreviations as in Table 1 (*AGP* Above ground parts, *ED* endophyte dependency, *NPP* net primary productivity, *UGP* under ground parts)

Hosts	Treatment	Fresh weight (g)		Dry weight (g)		NPP g/plant/day	ED
		AGP	UGP	AGP	UGP		
S.c.	*P.i.*	74.74 (±0.65)	9.26 (±0.15)	14.76 (±0.11)	2.13 (±0.23)	0.06	211.13
	Control	8.74 (±0.55)	6.26 (±0.35)	6.54 (±0.06)	1.46 (±0.06)	0.02	
W.s.	*P.i.*	152.53 (±0.76)	10.70 (±0.26)	63.03 (±0.15)	4.63 (±0.15)	0.23	671.90
	Control	19.07 (±1.1)	3.77 (±0.25)	8.67 (±0.35)	1.40 (±0.36)	0.12	

sis of as yet unidentified extracellular phytohormones by *P. indica* (Singh et al. 2000; Varma et al. 2001).

In conclusion, there was a significant increase in growth and yield of both *S. calva* and *W. somnifera* plants inoculated with *P. indica* relative to uninoculated controls. The differences in growth observed between inoculated and control plants may have been caused by greater absorption of water and mineral nutrients due to extensive colonization of roots by *P. indica*. The ability of *P. indica* to continue improving growth of *S. calva* and *W. somnifera* even during the hot March–June summer season (day temperature above 40°C) suggests that the fungus may improve drought tolerance of plants. However, this should be tested elsewhere in other soil conditions and geographical regions. The increases in fresh and dry-weights in treated *W. somnifera* and *S. calva* may be related to increased root P uptake. This assumption is based on the earlier report by Sudha et al. (1998), who found active translocation of phosphate in rice and transformed carrot roots on inoculation with *P. indica* (Varma et al. 2001). Our present findings underline the potential for using *P. indica* for large-scale cultivation of *W. somnifera* and *S. calva*.

Acknowledgements M.R. is grateful to Prof. Surendra Albert Brown, Principal, Danielson College for providing laboratory facilities and encouragement. Thanks are also due to the Department of Biotechnology and the Department of Science and Technology, Government of India for partial financial assistance.

References

Azcon R, Ocampo JA (1981) Factors affecting the vesicular-arbuscular mycorrhizal infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytol 87:677–685

Azcon-Aguilar C, Encina CL, Azcon R, Barea JM (1994) Effect of arbuscular mycorrhizae on growth and development of *Annona cherimola* micropropagated plants. Agric Sci Finland 3: 281–288

Bagyaraj DJ, Varma A (1995) Interaction between arbuscular mycorrhizal fungi and plants and their importance in sustainable agriculture in arid and semi-arid tropics. Adv Microb Ecol 14:119–142

Bailey NTJ (1995) Statistical methods in biology. Cambridge University Press, Cambridge, UK

Baylis GTS (1975) The magnoloid mycorrhiza and mycotrophy in root systems derived from it. In: Sanders FE, Mosser B, Tinker PB (eds) Endomycorrhizas. Academic, London, pp 373–389

Bhargava NC, Singh OP (1978). Fortege: an indigenous drug in common sexual disorders in males. Mediscope 21:140–141

Blechert O, Kost G, Hassel A, Rexer K-H, Varma A (1999) First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchid. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology, 2nd edn. Springer, Berlin Heidelberg New York, pp 683–688

Dey AC (1980) Indian medicinal plants used in ayurvedic preparations. Bishen Singh Mahendra Pal Singh, Dehradun

Dix NJ, Webster J (1995) Fungal ecology. Chapman and Hill, New York

Fortuna P, Citernesi S, Morini S, Giovannetti M, Loreti F (1992) Infectivity and effectiveness of different species of arbuscular mycorrhizal fungi in micropropagated plants of Mr S 2/5 plum rootstock. Agronomie 12:825–829

Franken P, Buetehorn B, Varma A (1998) *Piriformospora indica*, a cultivable root cell-infecting fungus promotes the growth of a broad range of plant species. In: Ahonen-Jonnarth U, Danell E, Fransson P, Karen O, Lindahl B, Rangel I, Finlay R (eds) Second International Congress on Mycorrhiza, Uppsala, Sweden, July 5–10

Gerdemann JW (1975) Vesicular-arbuscular-mycorrhizae. In: Torrey JG, Clarkson DT (eds) The development and function of roots. Academic Press, London

Gianinazzi S, Trouvelot A, Gianinazzi-Pearson V (1990) Role and use of mycorrhizas in horticultural crop production. Adv Hortic Sci 4:25–30

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

Kaefer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet 19:33–131

Menge JA, Johnson ELV, Platt RG (1978) Mycorrhizal dependency of several citrus cultivars under three nutrient regimes. New Phytol 81:553–559

Misra R (1968) Ecology work book. Oxford and IBH, New Delhi

Morte A, Grisela D, Honrubia M (1996) Effect of arbuscular mycorrhizal inoculation on micropropagated *Tetraclinis articulata* growth and survival. Agronomie 16:633–637

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Rajapakse S, Miller Jr JC (1988) Relationship between cowpea root systems and mycorrhizal dependency. Hortic Sci 23:568–570

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Sahay NS, Varma A (2000) Biological approach towards increasing the survival rates of micropropagated plants. Curr Sci 78: 126–129

Sahni YP, Srivastava DN (1993) *Withania somnifera*: an indigenous anti-ulcergenic drug. Indian J Ind Med 10:53–56

Sankaran JR (1984) Problem of male virility – an oriental therapy. J Natl Med Assoc 26:315–317

Schulthess FM, Faeth SH (1998) Distribution, abundances, and associations of the endophyte fungal community of *Arizona fescue* (*Festuca arizonica*). Mycologia 90:569–578

Singh A (2001) Intergeneric hybridization between a yeast strain and a plant growth-promoting endophytic fungus by isolation, fusion and regeneration of somatic protoplasts. PhD thesis, Jawaharlal Nehru University, New Delhi

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. Curr Sci 79:101–106

Sudha, Hurek T, Varma A (1998) Active translocation of phosphate (P32) 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, Uppsala, Sweden, July 5–10

Tripathi AK, Shukla YN, Kumar S (1996) Ashwagandha (*Withania somnifera*): a status report. J Med Aroma Plant Sci 18: 46–62

Varma A (1995) Arbuscular mycorrhiza fungi: the state of the art. Crit Rev Biotechnol 15:179–199

Varma A (1998) Mycorrhizae, the friendly fungi: what we know, what should we know and how do we know? In: Varma A (ed) Modern laboratory manual – Mycorrhizae. Springer, Berlin Heidelberg New York, pp 1–24

Varma A (1999) Functions and applications of arbuscular mycorrhizal fungi in arid and semi-arid soils. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology, 2nd edn. Springer, Berlin Heidelberg New York, pp 521–556

Varma A, Schuepp H (1995) Mycorrhizae, their application in micropropagated plantlets. Crit Rev Biotechnol 15:313–328

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. Appl Environ Microbiol 65:2741–2744

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 B (ed) Mycota IX. Springer, Berlin Heidelberg New York, pp 123–150

Verma S, Varma A, Rexer K-H, Kost G, Sarbhoy A, Bisen P, Buetehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 95:896–903