16 Co-Cultivation with Sebacinales

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16.1 Introduction

Mycorrhiza refers to an association or symbiosis between plants and fungi that colonize their roots during periods of active plant growth. The most common and prevalent, arbuscular mycorrhizal (AM) fungi, play an indispensable role in upgrading plant growth, vigour and survival by a positive impact on the nutritional and hydratic status of the plant and on soil health by increasing the reproductive potential, improving root performance and providing a natural defence against invaders including pests and pathogens (Newsham et al. 1995; Auge 2000; Borowicz 2001).

The majority of land plants live in mycorrhizal interaction with fungi, a symbiosis that has a strong impact on ecosystems, agriculture, flori-horticulture and forestry (Sanders 2003; Bidartondo et al. 2004; Koide and Mosse 2004; Pennisi 2004). The benefits of mycorrhizal associations arise from nutrient transport between the plant roots and fungal hyphae. The carbon source is transported from the plant to the fungus, whereas fungal hyphae serve as a fine link between the roots and the rhizosphere and improve the plant's supply of inorganic nutrients (Harrison 1999; Bücking and Heyser 2003; Herrmanns et al. 2004; Koide and Mosse 2004).

Applications of mycorrhizae in micropropagated plantlets are a boon for the micropropagation industry (Varma and Schüepp 1995). The key functions of AM co-cultivation can be summarized as follows: (1) improving root growth and

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plant establishment, (2) enhancing plant tolerance to (biotic and abiotic) stresses, (3) improving nutrient cycling, (4) enhancing plant community diversity.

16.2 Sebacinaceae – Novel Fungi

Bandoni (1984) revised the Tremellales and Auriculariales on the basis of ultrastructural, ontogenetic and ecological characters. The Sebacinaceae were transferred to his new concept of Auriculariales that then included taxa with septate basidia and continuous parenthesomes, but without a yeast stage. Weiß and Oberwinkler (2001) validated wide parts of Bandoni's (1984) concept of the Auriculariales in a molecular phylogenetic study using nuclear rDNA coding for the D1/D2 region of the large ribosomal subunit (LSU). Their molecular analysis confirmed the monophyly of the Sebacinaceae (including also *Craterocolla cerasi*, which fits the micromorphological concept of Sebacinaceae); however it also suggested that the Sebacinaceae form a separate lineage of Hymenomycetes that must be excluded from the Auriculariales.

Warcup and Talbot (1967) isolated heterobasidiomycetes that they identified from their sexual stages formed in axenic culture as *Sebacina vermifera* sensu stricto from the roots of Australian terrestrial orchids. Later such fungi were also isolated from pot-cultured ectomycorrhizae and arbuscular mycor-

Table 16.1 Recognized members of the Sebacinaceae

Fungus	Remark
Sebacina incrustans	Non-culturable[a]
S. epigaea	Non-culturable[a]
S. aff. epigaea	Non-culturable[a]
Tremelloscypha gelatinosa	Non-culturable[a]
S. dimitica	Non-culturable[a]
E. bulobasidium rolleyi	Non-culturable[a]
Craterocolla cerasi	Non-culturable[a]
Piriformospora indica	Culturable
Sebacina vermifera sensu stricto	Culturable
Sebacina sp.	Culturable

[a] Scientists have failed to culture these fungi on defined synthetic media

rhizae (Warcup 1988). Recently, using molecular methods like polymerase chain reaction (PCR), molecular cloning and sequencing, members of the Sebacinaceae have been shown to be involved in various mycorrhizal associations in the field: (1) orchid mycorrhizae (McKendrick et al. 2002; Selosse et al. 2002 a, b; Urban et al. 2003), (2) ectomycorrhizae (Berch et al. 2002). Since the remaining taxa of the Auriculariales sensu Bandoni (1984) are likely to be wood decomposers (Wells and Bandoni 2001), the mycorrhizal potential of the Sebacinaceae seems a good ecological features to separate members of this from other, morphologically quite similar heterobasidiomycetes that belong to the Auriculariales. However, sebacinoids were demonstrated recently to be ectomycorrhizal (Selosse et al. 2002a). Observations on ectomycorrhizae and basidiomes suggest that species of Sebacinaceae are fairly common mycobionts in various ectomycorrhizal plant communities (Urban et al. 2003). The phylogenetic position of the Sebacinaceae within the Basidiomycota gives an overview of phylogenetic relationships inside this subgroup of Hymenomycetes for which the new Sebacinales is proposed (Garnica et al. 2003; Michael Weiß, personal communication). Fungal strains included in the Sebacinaceae are given in Table 16.1.

16.3 Host Spectrum

Members of the Sebacinaceae were observed to be associated with a large number of mono- and dicotyledonous plants (Table 16.2), inducing pronounced growth promotional effects (Varma et al. 2001), with the exception of the plants belonging to the Cruciferaceae and some plants belonging to the Chenopodiaceae and Amaranthaceae (Read 1999; Varma et al. 1999, 2001; Singh et al. 2003b). Literature suggests that the members of these groups normally do not form associations with AM fungi (Denison et al. 2003). Under in vitro conditions, *P. indica* and *S. vermifera* sensu stricto were demonstrated to interact with the root system of cruciferous and chenopodaceous plants, viz. mustard (*Brassica junaceae*), cabbage (*Brassica oleracea* var. *capitata*; Kumari et al. 2003), *Arabidopsis thaliana* (Pham et al. 2004a) and spinach (*Spinacia oleracea*). A report indicated the ability of *P. indica* to colonize the rhizoids of a liverwort (bryophyte), and the thalli failed to grow under in situ conditions in the absence of this fungus (Varma et al. 2000, 2001; Pham et al. 2004a). *P. indica* was further shown to form associations with terrestrial orchids such as *Dactylorhiza purpurella* (Stephs.) Soo, *D. incarnate* L. Soo, *D. majalis* (Rchb. F.) Hunt & Summerh. and *D. fuchsia* (Druce) Soo (Blechert et al. 1999; Varma et al. 2001; Pham et al. 2004a; Prasad et al. 2005).

Table 16.2 Plant interactions tested with members of Sebacinaceae. Data is based on the root colonization analysis in vivo and in vitro (c.f. Varma et al. 2001; Singh et al. 2003a, b)

Hosts	
Acacia catechu (L.f.) Willd (black catechu)	*Glycine max* L. Merr. (soybean)
Acacia nilotica (L.) Willd (gum)	*Nicotiana tabaccum* L. (tobacco)
Abrus precatorius L. rosary pea (precatory bean)	*N. attenuata* L. (mountain tobacco)
Adhatoda vasica L. syn. (malabar nut)	*Oryza sativa* L. (rice)
Aneura pinguis L. Dumort. (liverwort)	*Petroselinum crispum* L. (curly parsley)
Arabidopsis thaliana L. Heynh. (mouse ear cress)	*Pisum sativum* L. (pea)
Artemisia annua L. (chinese wormwood)	*Populus tremula* L. (aspen)
Azadirachta indica A. Juss (neem)	*P. tremuloides Michx.* (clone Esch5; quaking)
Bacopa monniera L. Wett. (brahmi)	*Prosopis chilensis* Stuntz sys. (chilean mesquite)
Cassia angustifolia Senna Patti (gallow grass hemp)	*P. juliflora* (Swartz) DC. (honey mesquite)
Chlorophytum borivillianum Baker (musli)	*Quercus robur* L. (clone DF 159; oak)
Ch. tuberosum Baker (mexican orange)	*Setaria italica* L. (thumb millet)
Cicer arietinum L. (chick pea)	*Solanum melongena* L. (egg plant)
Coffea arabica L. (English coffee)	*Sorghum vulgare* L. (millet)
Cymbopogon martinii Staph Van Motia (palmarosa)	*Spilanthes calva* DC (clove)
Dactylorhiza fuchsi Druce (Soo') (spotted orchid)	*Tectona grandis* Linn. f. (teak)
D. incarnata L. Soo' (early marsh orchid)	*Terminalia arjuna* L. (Arjun tree/stembark)
D. maculata L. Verm. (Northern marsh orchid)	*Tephrosia purpurea* L. Pers. (sarphunkha/purpurea)
D. majalis Rchb. f. (broad leaved marsh orchid)	*Withania somnifera* L. Dunal (winter cherry)
D. purpurella (Steph's) Soo' (lady orchid)	*Zea mays* var white (maize)
Daucus carota L. Queen Anne's-lace (carrot)	*Zizyphus nummularia* Burm. fil. (jujube)
Delbergia sisso Roxburg (rosewood)	

16.4 Functions of the Sebacinaceae

Scientists from the Amity University Uttar Pradesh, Noida, have screened a novel endophytic fungus, *Piriformospora indica*, which mimics the capabilities of a typical AM fungus. *P. indica* is a recently isolated root-interacting fungus, related to the Hymenomycetes of the Basidiomycota (Verma et al. 1998). In contrast to AM fungi, it can be easily cultivated in axenic culture where it produces chlamydospores (Peškan-Berghöfer et al. 2004; Oelmüller et al. 2005; Shahollari et al. 2005). The fungus is able to associate with the roots of various plant species in a manner similar to mycorrhiza and promotes plant growth (Varma et al. 1999, 2001; Singh et al. 2002a, b, 2003a; Oelmüller et al. 2004; Pešken-Berghöfer et al. 2004; Pham et al. 2004a; Shahollari et al. 2005). Pronounced growth promotional effects were also seen with terrestrial orchids (Blechert et al. 1999). The fungus can easily be cultivated on a number of synthetic and complex media (Hill and Käfer 2001; Pham et al. 2004b).

P. indica tremendously improves the growth and overall biomass production of diverse hosts, including legumes, medicinal and economically important plants (Varma et al. 1999, 2000). The plants tested in the laboratory conditions as well as in extensive field trials were *Bacopa monieri*, *Nicotiana tobaccum* (Sahay and Varma 1999, 2000), *Artemisia annua*, *Petroselinum crispum* (Varma et al. 1999), *Azadirachta indica* (Singh et al. 2002a, b, 2003a), *Tridex procumbans*, *Abrus precatorius* (Kumari et al. 2004), *Chlorophytum borivilianum* (Pham et al. 2004a), *Withania somnifera* and *Spilanthes calva* (Rai et al. 2001) and *Adhatoda vasica* (Rai and Varma 2005). *P. indica* promotes the antifungal potential of the medicinal plant *Spilanthus calva* due to an increase in spilanthol content after interaction (Rai et al. 2004). *P. indica* promises to be an excellent agent for the biological hardening of tissue culture-raised plants as the fungus rendered more than 90% survival rate of the transferred plantlets of these plants and, by excessive root proliferation and induction of secondary rootlets, protecting them from “transplantation shock” and potent root pathogens (Singh et al. 2002a, b, 2003b; Varma et al. 2000). Therefore, this fungus has promise as a boon for the plant industries (Hazarika 2003; Singh et al. 2003a).

Among the compounds released in root exudates, flavonoids are found to be present in *P. indica*. Flavonoids have been suggested to be involved in the stimulation of pre-contact hyphal growth and branching (Gianinazzi-Pearson et al. 1989; Siqueira et al.1991), which is consistent with their role as signalling molecules in other plant–microbe interactions (Giovannetti and Sbrana 1998). Cell wall-degrading enzymes like CMCase, polygalactouronase and xylanase were found in significant quantities both in the culture filtrate and in roots colonized with *P. indica*.

P. indica showed a profound effect on disease control when challenged with the virulent root and seed pathogen *Gaeumannomyces graminis*. *P. indica* completely blocked growth of this pathogen. This indicates that *P. indica* acts as a

potential agent for biological control of root diseases; however the chemical nature of the inhibitory factor is still unknown (Varma et al. 2001).

P. indica has been reported to induce resistance to fungal diseases in the monocotyledonous plant barley, along with tolerance to salt stress without affecting plant productivity (Waller et al. 2005). The beneficial effect on the defence status is detected in distal leaves demonstrating a systemic induction of resistance. The systemically altered "defence readiness" is associated with an elevated antioxidative capacity due to an activation of the glutathione–ascorbate cycle and an overall increase in grain yield. Interaction with *Populus* Esch5 revealed that *P. indica* could be directed in its physiological behaviour from mutualistic to antagonistic by specifically designed cultural conditions (Kaldorf et al. 2005), hence making it a potential model system to study plant–microbe interactions. It provides a promising model organism for the investigation of beneficial plant–microbe interactions, and enables the identification of compounds, which may improve plant growth and productivity and maintain soil productivity. The various multifunctional roles of *P. indica* are outlined in Fig. 16.1.

16.5 Eco-Functional Identity

Members of the Sebacinales, *P. indica* and *S. vermifera* colonize the root cortex and forms inter- and intracellular hyphae. Within the cortical cells, the fungus often forms dense hyphal coils or branched structures, intracellularly. *P. indica* also forms spore- or vesicle-like structures within or between the cortical cells.

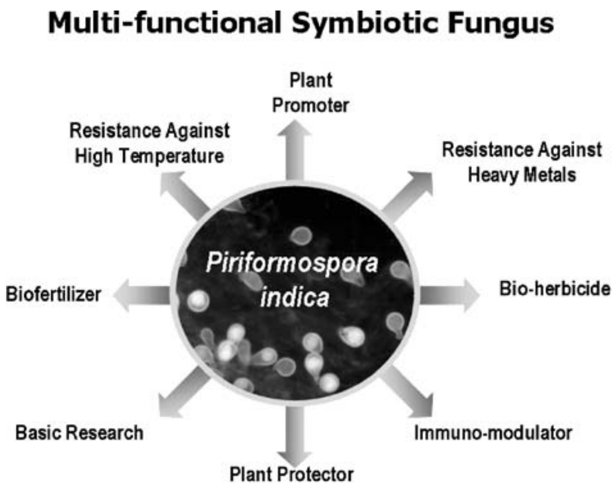

Fig. 16.1 Multifunctional role of *P. indica*

Like AM fungi, hyphae multiply within the host cortical tissues and never traverse through the endodermis. Likewise, they also do not invade the aerial portion of the plant (stem and leaves).

The characteristic features of *P. indica* are the following:

- axenically cultivable on synthetic media,
- no clamp connections,
- anastomosis occurs frequently,
- hypha–hypha aggregation often observed,
- no hyphal knots,
- simple septum with dolipores and continuous, straight parenthosomes (Fig. 16.2 inset),
- chlamydospores 16–25 μm in length, 10–17 μm in width,
- 8–25 nuclei per spore.

The fungus promises to serve as the substitute of AM fungi to overcome the long-standing enigma of science. The functional similarities with AM fungi are the following:

- broad and diverse host spectrum,

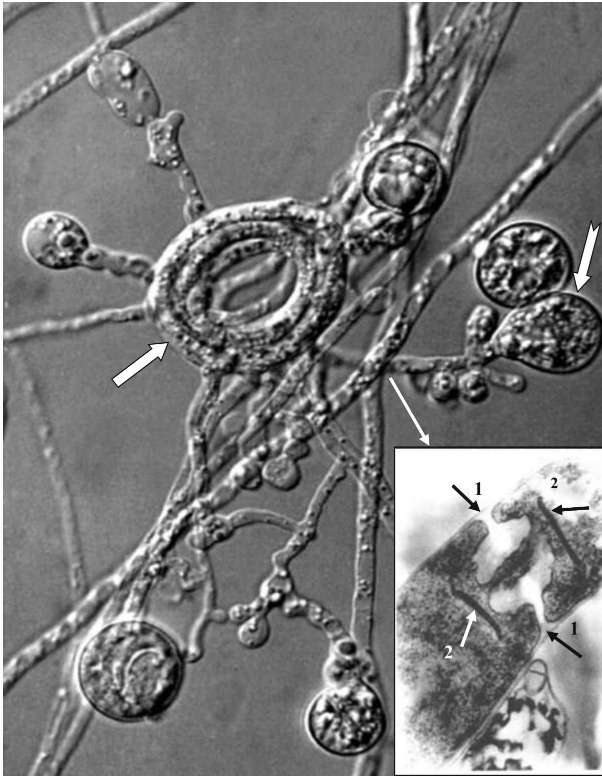

Fig. 16.2 *P. indica*: an overall view of the typical growth and differentiation of hyphae on solidified Käfer medium (the *white arrow* shows the hyphal coil and pear-shaped spore). *Inset*: a magnified view showed the dolipore and parenthosomes of *P. indica* (a section of hypha was observed in electron-transparent material): the *small white arrow* indicates the dolipore and the *black arrows* indicate the continuous parenthosomes. This septal pore is typical for Hymenomycetes

- hyphae extramatrical, inter- and intracellular,
- hyphae never invade the endodermis,
- chlamydospores in soil and within cortical tissues,
- sexual stages not seen,
- positive phytopromotional effects on tested hosts,
- phosphorus mobilizer,
- phosphorus transporter,
- tool for biological hardening of micropropagated plantlets,
- potent biological control agent against root pathogens.

16.6 Axenic Co-Cultivation of Sebacinaceae

Circular agar discs (about 4 mm in diameter) infested with spores and actively growing hyphae of *P. indica* were placed onto Petri dishes (90 mm, disposable; Tarson, India) containing solidified Hill and Kafer medium. Inoculated Petri dishes (90 mm, disposable) were incubated in an inverted position for 7 days at 28±2 °C in the dark. Usually 4–5 fully-grown fungus agar discs (4 mm in diameter) were inoculated into each 500-ml Erlenmeyer flask containing 250 ml of Hill and Kafer broth. Flasks were incubated at 28±2 °C, at constant shaking at 100 rpm on a rotary shaker. The same procedure was performed for *S. vermifera* sensu stricto and *Sebacina* sp.

16.6.1 Procedure

1. Circular agar discs (about 4 mm in diameter) infested with spores and actively growing hyphae of *P. indica* are placed onto Petri dishes (90 mm, disposable) containing solidified Hill and Kafer medium (Fig. 16.3a).
2. Inoculated Petri dishes (90 mm, disposable) are then incubated in an inverted position for 7 days at 28±2 °C in the dark.
3. Four or five fully grown fungus agar discs (4 mm in diameter; Fig. 16.3b) are inoculated into each 500-ml Erlenmeyer flask containing 250 ml of *Aspergillus* broth.
4. Flasks are incubated at 28±2 °C, at constant shaking at 100 rpm on a rotary shaker (Fig. 16.3c).

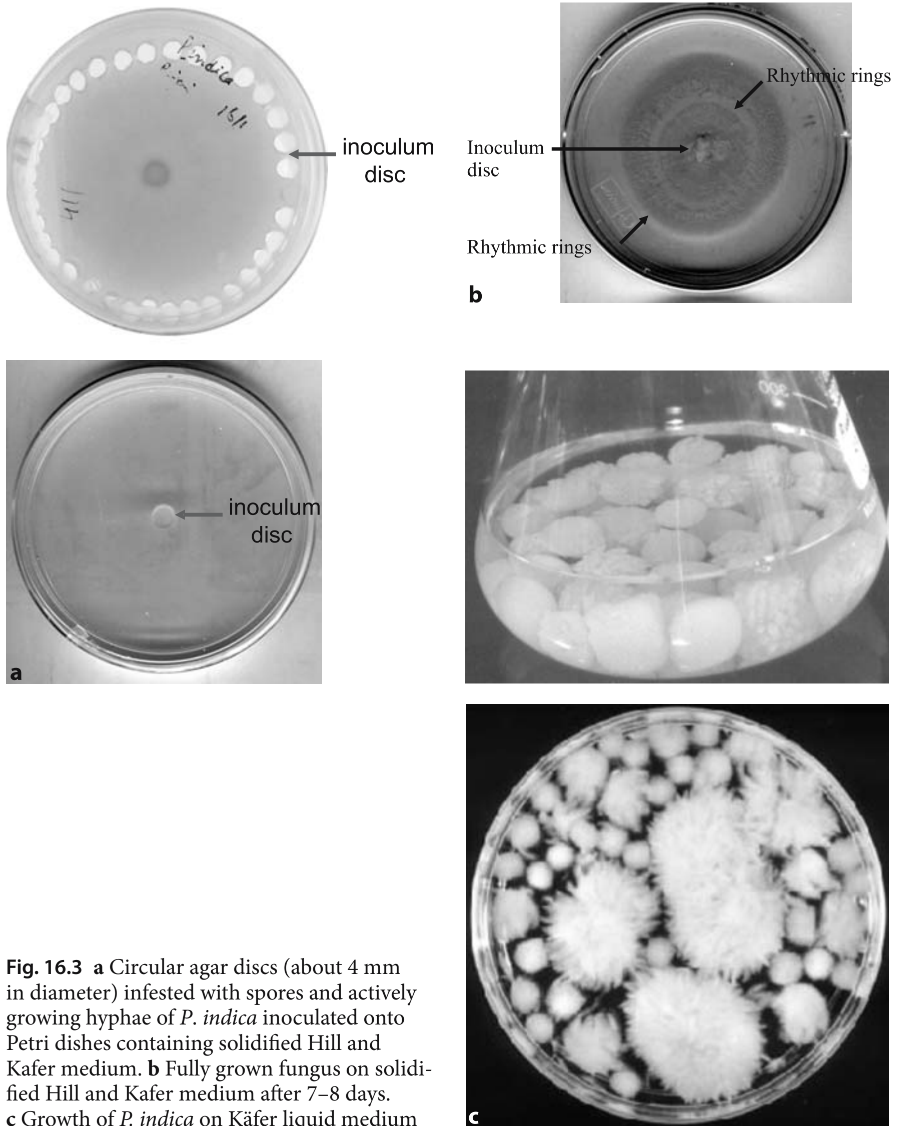

Fig. 16.3 **a** Circular agar discs (about 4 mm in diameter) infested with spores and actively growing hyphae of *P. indica* inoculated onto Petri dishes containing solidified Hill and Kafer medium. **b** Fully grown fungus on solidified Hill and Kafer medium after 7–8 days. **c** Growth of *P. indica* on Käfer liquid medium

16.6.2 Protocol

1. Hold the mother culture of *P. indica* grown on Hill and Kafer medium (Hill and Käfer 2001) inside a laminar flow hood.
2. Make the discs by using the bottom of a sterile glass Pasteur pipette measuring about 4 mm in diameter.
3. Inoculate one disc per Petri plate fortified with Hill and Kafer medium containing 1% agar.
4. Wrap the Petri plates with paraffin tape to avoid any contamination.
5. Incubate the Petri plates at 28±2 °C.
6. Growth normally commences on the third day and, after 12 days, the fungus completely covers the surface of the agar plate (Fig. 16.3b).

16.7 Media Compositions

1. The Hill and Kafer medium composition is given in Table 16.3.
2. For modified Hill and Kafer medium (Varma et al. 2001), the medium composition is the same, except that the quantities of yeast extract, peptone and casein hydrolysate are reduced to one-tenth in quantity.
3. Glucose asparagine agar (for Actinomycetes):

Table 16.3 Composition of Hill and Kafer medium (Hill and Kafer 2001). The pH is adjusted to 6.5 with 1 N HCl/NaOH. All stocks are stored at 4 °C except vitamins, which are stored at –20 °C. In broth culture, agar is excluded

Constituent	Concentration (g/l)
Glucose	20.0
Peptone	2.0
Yeast extract	1.0
Casamino acid	1.0
Vitamin stock solution	1.0 ml
Macroelements from stock	50 ml
Microelements from stock	2.5 ml
Agar	10
$CaCl_2$, 0.1 M	1.0 ml
$FeCl_3$, 0.1 M	1.0 ml

Table 16.3 *(continued)*

Constituent	Concentration (g/l)
Macroelements (major elements) stock (g/l)	
$NaNO_3$	120.0
KCl	10.4
$MgSO_4.7H_2O$	10.4
KH_2PO_4	30.4
Microelements (trace elements) stock (g/l)	
$ZnSO_4.7H_2O$	22.0
H_3BO_3	11.0
$MnCl_2.4H_2O$	5.0
$FeSO_4.7H_2O$	5.0
$CoCl_2.6H_2O$	1.6
$CuSO_4.5H_2O$	1.6
$(NH_4)_6Mo_7O_{27}.4H_2O$	1.1
Na_2EDTA	50.0
Vitamins (%)	
Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

Constituent	Concentration (g/l)
Glucose	10
Asparagine	0.5
K_2HPO_4	0.5
Distilled water	1000 ml
Agar	15
pH at 25 °C	6.8±0.2

Directions: ingredients are suspended in 1000 ml of distilled water. Dissolve by boiling completely. Distribute in flasks and sterilize in the autoclave at 15 psi pressure (103 kPa) at 121 °C for 15 min

4. Hoagland solution (Hoagland and Arnon 1938):

Constituent	Concentration (g/l)
Macro-nutrients:	
$MgSO_4.7H_2O$	490
$Ca(NO_3)_2.4H_2O$	492
KNO_3	1002
$CuSO_4\,5H_2O$	230
Micro-nutrients:	
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
H_3BO_3	2.86
$CuSO_4.5H_2O$	0.08
$NaMoO_4$	0.09
Iron source	31.0

Directions: all ingredients are dissolved separately in double-distilled water and then mixed (pH = 6.7)

5. Malt extract medium (Gallowey et al. 1962):

Constituent	Concentration (g/l)
Malt extract	30
Mycological peptone	5
Agar	15
pH	5.4

6. Malt yeast extract medium:

Constituent	Concentration (g/l)
Yeast extract	3
Malt extract	3
Peptone	5
Dextrose	10
pH (25 °C)	6.2±0.2

7. Malt yeast extract agar: add 2% (w/v) agar to the above malt yeast extract medium.

8. Modified Melin–Norkrans (MMN) medium (Johnson et al. 1957):

Constituent	Concentration
NaCl	0.4 mM
KH_2PO_4	3.7 mM
$(NH_4)_2HPO_4$	2.0 mM
$CaCl_2$	0.3 mM
$MgSO_4$	0.6 mM
$FeCl_3$	3.6 mM
Thiamine hydrochloride	0.2 mM
Trypticase peptone	0.1% (w/v)
Glucose monohydrate	1.0% (w/v)
Malt extract	5.0% (w/v)
Trace elements from stock	10 ml/l

a. Stock solution of trace elements:

Constituent	Concentration
KCl	0.2 M
H_3BO_3	0.1 M
$MnSO_4.H_2O$	22.0 mM
$ZnSO_4$	8.0 mM
$CuSO_4$	2.1 mM
pH	5.8

9. MMN agar medium: add 1.2% (w/v) agar to the above MMN medium.
10. Plate count agar (APHA 1978):

Constituent	Concentration (g/l)
Trypton	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
pH (25 °C)	7.0±0.2

Directions: suspend about 23.5 g of plate count agar in 1000 ml of distilled water. The medium is completely dissolved by boiling and is then sterilized at 15 psi pressure at 121 °C for 15 min.

11. Potato dextrose agar (PDA; APHA 1978):

Constituent	Concentration (g/l)
Potato peel	200
Dextrose	20
Agar	15
Distilled water	1000
pH (25 °C)	5.6±0.2

Directions: the periderm (skin) of potatoes (200 g) is peeled off, cut into small pieces and boiled in 500 ml of water, until a glass rod easily penetrates them. After filtration through cheesecloth, dextrose is added. Agar is dissolved and the required volume (1 l) is made up by the addition of water. The medium is autoclaved at 15 psi pressure at 121 °C for 20 min

12. Water agar (WA):

Constituent	Concentration (g/l)
Daichin agar	7 (0.7%)

13. 20% Knop solution:

Constituent	Concentration (g/l)
Saccharose	20.0 (2.0%)
Daichin agar	8.0 (0.8%)
Vitamin B5 (Gamborg and Phillips 1996)	1.0
Stock solution I	2.0
Stock solution II	2.0
Stock solution III	2.0
Stock solution IV	0.4
Stock solution V	0.2

Adjust pH to 6.4 with 1 N KOH

14. Composition of stock solutions I–V for Knop solution:

Stock solution	Constituent	Concentration (g/l)
Stock solution I	KNO_3	121.32
	$MgSO_4.7H_2O$	19.71
Stock solution II	$Ca(NO_3)_2.4H_2O$	120.0
Stock solution III	KH_2PO_4	27.22
Stock solution IV	FeNaEDTA	7.34
Stock solution V	H_3BO_3	2.86
	$MnCl_2$	1.81
	(or $MnCl_2.4H_2O$)	(2.85)
	$CuSO_4.5H_2O$	0.073
	(or $CuSO_4.2H_2O$)	(0.05)
	$ZnSO_4.7H_2O$	0.36
	$CoCl_2.6H_2O$	0.03
	H_2MoO_4	0.052
	(or $Na_2MoO_4.2H_2O$)	(0.0775)
	NaCl	2.0

16.8 Seed Surface Sterilization and Germination

Maize seeds are soaked in sterile water overnight and surface-sterilized by washing with 90% ethanol for a few seconds and either with 0.01% mercuric chloride solution for 10 min or with 4% (v/v) NaOCl for 15 min, then washed five times with sterile distilled water and finally rinsed with 70% (v/v) ethanol for 30 s. This is followed by a quick treatment with 15% (v/v) NaOCl; chemicals adhered are removed by repeated rinsing with sterile distilled water (or a better alternative method can be used as described in Section 16.8.1; Gamborg and Phillips 1996). The seeds are kept 1 cm apart in a sterile Petri dish layered with germinating paper or aseptically transferred to water agar plates (0.7% agar) and left for germination at 25±2 °C for 4 days in the dark.

16.8.1 Protocol for Seed Surface Sterilization

1. Collect desired quantity of seeds.
2. Soak in sterilized distilled water overnight.
3. Treat with 70% ethanol for 30 s with stirring.
4. Wash three times with sterile distilled water to remove traces of ethanol.
5. Wash with 1.5% NaOCl solution for 20 min with stirring
6. Wash three times with sterilized distilled water.
7. Wash with 15% NaOCl for 20 s.
8. Wash six times with distilled water to remove traces of NaOCl.

Garden soil is sterilized by autoclaving three times at 121 °C at 15 psi pressure (103 kPa), at intervals of 48 h. Sand is acid-treated in 10% HCl overnight and washed in running tapwater until the pH becomes neutral. Sterile soil and acid-washed sand are dried in a hot-air oven. Soil and sand are mixed in the ratio of 3:1 for filling the pots.

16.8.2 Inoculum Placement in the Pots

Live inoculum of *P. indica* is required. This contains spores and fungal hyphae. In the pot, a soil base is added first, up to one-third of the depth of pot. Then live inoculum is layered over it. Above this layer, one layer of soil base is added to sandwich the inoculum between the layers of soil base. For the inoculation of *P. indica*, mycelium is mixed in a small amount of sterile soil and then spread as above, in a sandwich model at the rate of 1%.

Surface-sterilized seeds are transferred to the pots. When the plants reach 2–3 cm, they are then treated with Hoagland solution. The morphological features of each plant are observed and recorded at weeks 2, 4 and 8.

16.8.3 Results

16.8.3.1 *P. indica* – Photobiont Interaction

Fungus-treated plants were compared with untreated control plants in terms of morphological and anatomical characteristics. As an impact of *P. indica*, the

treated plants showed early germination in comparison with the uninoculated control. After 30 days, prominent differences were seen. *P. indica*-treated plants become longer with more nodes and leaves than the control.

16.8.3.2
Growth Conditions

Pots were placed in a greenhouse maintained at 30±2 °C, 16 h photoperiod (1000 lux) and 75% relative humidity for four months. The plants were fertilized with 10% strength Hoagland solution (Hoagland and Arnon 1938) on every alternate week, consisting of phosphorus and devoid of phosphorus nutrients. Plants were irrigated with sterile tapwater on every alternate day to maintain a relative moisture of about 60%.

16.8.3.3
Growth Parameters

1. Aerial length: the height of each plant was measured at intervals of 14, 28 and 42 days. Experiments were recorded in triplicate.
2. Aerial biomass: each endophyte-inoculated plant was carefully taken in triplicate from the pots for fresh and dry quantification at intervals of 14, 28 and 42 days. Plants were wiped with tissue paper and air-dried for fresh weight. Later they were dried at 80 °C for 12 h in an air-circulation Memmert-type oven. Samples were desiccated at room temperature before weighing on a Mettler balance (AE 160).
3. Underground length: underground parts were thoroughly washed under running tapwater to remove the adhering soil particles. The length of the underground part was measured in triplicate readings.
4. Underground biomass: after excessive washing, the moisture was blotted out with filter paper, then air-dried and weighed for fresh weight on a Mettler balance (AE 160).
5. Endophyte dependency: the endophyte dependency (ED) of *Zea mays* L. var white was determined using the formula given by Gerdemann (1975), which was modified by Plenchette et al. (1983) to give a percent increase of yield relative to that of mycorrhizal plants. This results in a figure between 0% and 100% rather than an unlimited percent increase:

   ED = (Parameter with mycorrhiza – parameter without mycorrhiza)/(Parameter with mycorrhiza) ×100
   ED was used instead of mycorrhiza dependency (MD) to designate endophyte dependency.

16.9
Comparative Study on Plant Growth with Treated Endosymbionts

Both *P. indica* and *Sebacina vermifera* sensu stricto exhibited the highest positive growth-promoting effect on maize plants, as evidenced by better aerial length (above ground), enhanced and healthier foliage and a well developed rooting system, as compared with other endophytic strains. *S. vermifera* sensu stricto showed a little less growth-promoting effect than *P. indica*.

Mycorrhiza dependency (MD) was used as an index to compare the receptivity of different plant species to AM fungi (Gerdemann 1975; Plenchette et al. 1983). This can also be used for other endophytes, such as *P. indica* and *S. vermifera* sensu stricto. In the present study, ED was used instead of MD, as the test organisms do not develop a typical mycorrhizal association. *P. indica* showed the highest ED over the other related endophytes. The more intense root proliferation in treated plants observed in the present experiments might be due to the synthesis of as yet unidentified extracellular phytohormones by mycobionts (Singh et al. 2000; Varma et al. 2001). The ED value was 211.13 for *Spilanthes calva* and 671.90 for *Withenia somnifera*. These data suggest that *P. indica* has a greater influence on the growth of *W. somnifera* than on that of *S. calva* (Rai et al. 2001). The ED of a host plant can be altered by factors such as soil type and the soil P content of mycorrhizal species (Azcon and Ocampo 1981; Menge et al. 1978). Amongst the reasons proposed for the differences in ED in different plants or varieties of the same species, Baylis (1995) reported that root-hair length and root thickness could determine the ED level. Rajapakse and Miller (1988) observed that the average length of fine roots was negatively correlated with ED in cowpea.

16.10
In Vivo Co-Cultivation of Sebacinales

The Sebacinaceae members were also inoculated into sterile soil in polyethylene or earthenware pots in five replicates, using 0.5 kg capacity pots for mass cultivation. The soil was autoclaved thrice on alternate days and air-dried. Riverbed sand was soaked in 10% HCl overnight and then washed under running tap-water until the pH reached neutrality. An air-dried mixture of soil and sand in the ratio of 3:1 (Feldmann and Idczak 1994) was used as substratum. Pots were also surface-sterilized by 70% ethanol and were then half-filled with this mixture and the inoculum was layered over it. Five holes were made into each pot and into each hole approximately 1 g of endomycorrhizal inoculum was added (80 spores/fungal propagules per 10 g soil). Five germinated seeds of 10 mm

length were placed 1–2 cm above the inoculum layer in the marked holes in each pot (Fig. 16.4a, b). The pots were maintained in an environmentally controlled greenhouse at 25±2 °C with 16 h light/8 h dark and relative humidity 60–70%, with a light intensity of 1000 lux. Roots were checked for colonization after 15–20 days. The soil cultures, along with the root propagules obtained after four months, were stored in a cold room for further use. Root pieces with spores and hyphal fragments can be used as a live propagule (inoculum) for experiments or to introduce fungi into soils. Similarly, for comparative photomycobiont growth of *P. indica* and *S. vermifera* sensu stricto, a disc (4 mm diameter) of inoculum infested with hyphae and spores was taken per plant.

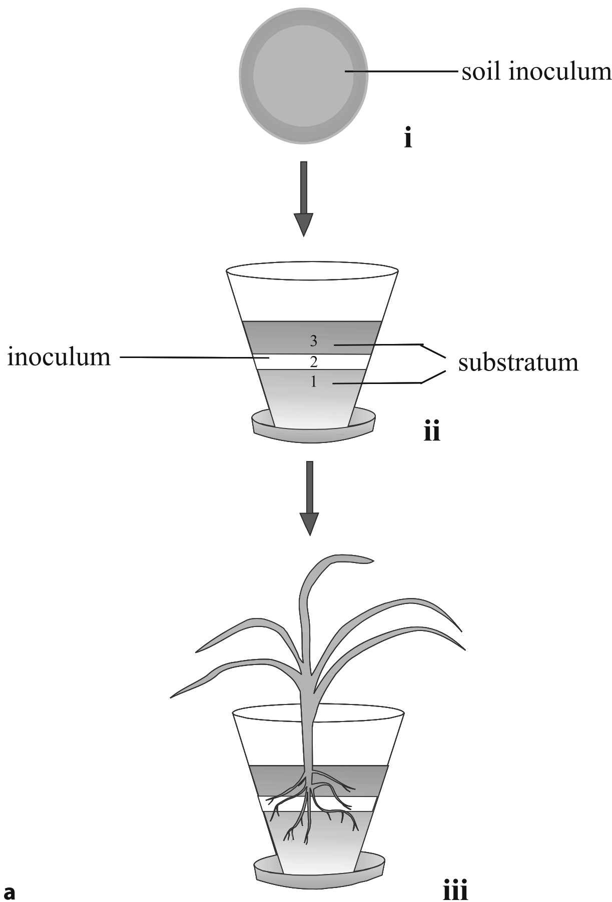

Fig. 16.4 **a** Polyethylene pots (0.5 kg capacity) contained an autoclaved sand and soil mixture (1:3) at pH 7.0. *Stage i* Soil inoculum consisting of spores, hyphae and colonized root propagules. *Stage ii* Sandwich of 1 cm layer of inoculum. *Stage iii* Micropropagated plantlets were plated up to the second layer in an upward direction. A little sterile tap water was gently sprinkled to moisten the upper soil layer **b** *see next page*

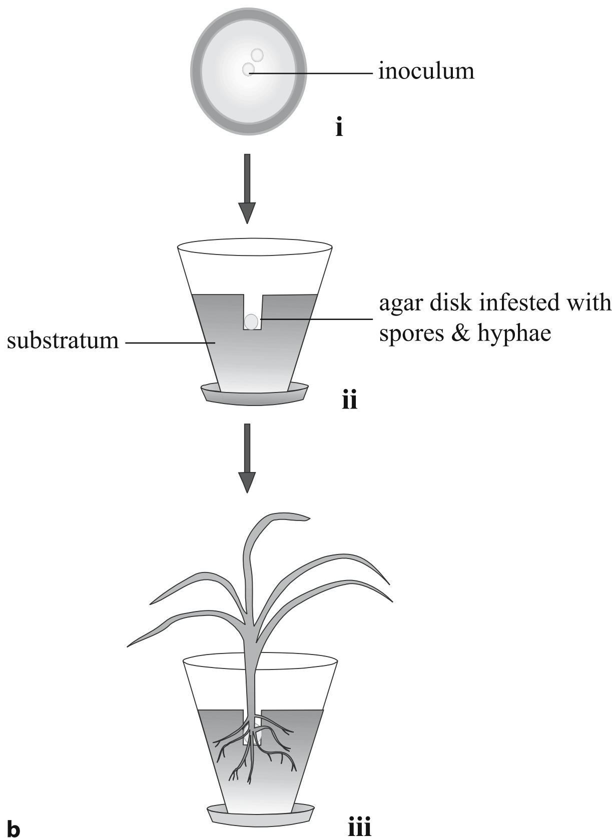

Fig. 16.4 *(continued)* **b** Polyethylene pots (0.5 kg capacity) contained an autoclaved sand and soil mixture (1:3) at pH 7.0. *Stage i* Culture inoculum in Petri dish consisting of spores and hyphae. *Stage ii* A hole was made in the centre of the pot, up to 2 cm deep, with the help of a surface-sterilized, specially designed plastic rod. An agar disc (4 mm diameter) infested with spores and hyphae was placed in the hole. *Stage iii* Micropropagated plantlets were inserted into the hole in an upward direction and the top was covered with the same substratum. A little sterile tap water was gently sprinkled to moisten the upper soil layer

16.11 Conclusions

Members of the Sebacinaceae have been found to be associated with a large number of mono- and dicotyledonous plants. Their interactions have shown growth promotion in diverse plant genera. *P. indica* and *S. vermifera* are root endosymbionts that can be considered as model organisms to study the hidden mystery of mycorrhizal world, since these fungi mimic the AM fungal characters.

The axenic cultivability of Sebacinales members *P. indica* and *S. vermifera* makes them ideal tools for further biotechnological exploitation. They serve as excellent organisms for biotechnological applications in the fields of agriculture, forestry, flori-horticulture, viticulture and arboriculture. They can also be used for the synthesis of herbicides, weedkillers, pesticides and several enzymes of industrial importance. Functionally, co-cultivation with *P. indica* not only promotes plant growth but also increases the plant's active constituents and enhances disease resistant. It is also an excellent biological hardening agent and the fungus renders an above 90% survival rate in tissue culture transplantation plants. Axenic cultivation of *P. indica* is very simple and the fungus can be normally multiplied on a variety of cheap media within a very short time and can be produced on a large scale. The axenically produced fungal inoculum can be directly used for co-cultivation under greenhouse and field conditions.

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