Proc Indian Natn Sci Acad **80** No. 2 June 2014 Spl. Sec. pp. 429-441

DOI: 10.16943/ptinsa/2014/v80i2/55119

Review Article

Functions of Novel Symbiotic Fungus - *Piriformospora indica*

A VARMA*, K SOWJANYA SREE, M ARORA, R BAJAJ, R PRASAD and A C KHARKWAL
Amity Institute of Microbial Technology, Amity University, Sector 125, Noida 201 303, Uttar Pradesh, India

(Received on 18 January 2013; Revised on 13 May 2013; Accepted on 29 July 2013)

A unique endophytic fungus, *Piriformospora indica*, isolated from the hot Thar desert, shows beneficial symbiotic associations with a wide range of host plants. This fungus promotes growth of the host plant in terms of seed germination, seedling growth, early flowering, enhanced fruiting and increase in the content of value addition products e.g. secondary metabolites. One of the possible mechanisms which explain these benefits is fungus-facilitated increase in the uptake of nutrients and minerals. Fungal inoculation also protects the plant from various abiotic and biotic stresses. Although the fungus was isolated from hot sand dunes, it equally works in the cold deserts of Leh-Ladakh. The interaction of this root endophyte with other Plant Growth Promoting Rhizobacteria was also investigated. To enhance the shelf life of the fungus and for its wide application by the farmers, a powder formulation has been developed with the registered trademark, Rootonic. The formulation has shown a very positive impact on plant productivity during field trials in Northern India.

Key Words: ***Piriformospora indica;*** **Rootonic; Plant Growth Promotion; Plant Growth Promoting Rhizobacteria**

Introduction

Piriformospora indica is a root-colonizing endophytic fungus beneficial to its host plants. It has a wide host range that includes those belonging to Bryophyta, Pteridophyta, Gymnosperm and Angiosperm [1-8]. Morphologically, the fungal hyphae are thin walled, hyaline, irregularly septated and form pear shaped chlamydospores. These chlamydospores are autofluorescent [8]. The fungus colonizes rhizodermis and cortical zones of the root, both inter- and intracellularly and does not invade the endodermis and aerial parts of the plant. The endophyte promotes nutrient uptake, and also confers resistance against biotic (insects and pathogenic microorganisms) and abiotic stresses (drought, salinity, heat and cold). Further, it stimulates excessive production of biomass, early flowering and seed production in its host plants [9, 10, 11]. In this review, an effort has been made to summarize and highlight important features of this fungus and its possible biotechnological applications.

The fungus *P. indica* was originally isolated from the roots of xerophytes growing in the Indian Thar desert [9, 12]. *P. indica* is a basidiomycete, which resembles arbuscular mycorrhizal fungi in many aspects, however, is categorised in the new family Sebacinaceae and the new order Sebacinales-Glomeromycota [13, 14]. Interestingly, *P. indica* can be cultured axenically [1, 3]. It colonizes roots in an endophytic fashion without any host specificity [1] promoting uptake of nutrients like phosphorus and nitrogen [10, 18]. Further, it allows plants to survive under water, temperature and salt stresses, imparts resistance to toxins, heavy metals and pathogenic organisms and stimulates growth, early flowering and seed production in its hosts. The wide host range includes bryophytes (*Aneura pinguis*), pteridophytes (*Pteris ensiormis*), gymnosperms (*Pinus halepensis*), and a large number of angiosperms (e.g. *Arabidopsis thaliana, Artemesia annua, Curcuma longa, Hordeum vulgare*). It was found to have a remarkable bio-hardening effect when inoculated with micropropagated plants [15]. However, genetically

*Author for Correspondence: E-mail: ajitvarma@amity.edu

modified *Populus* Esch5 cultured on WPM medium, having ammonium as the major source of nitrogen, caused the leaves in contact with the fungus to become brownish and bleached. the tissue culture raised genetically modified *Populus* Esch5 when grown on WPM medium having ammonium as the major source of nitrogen, the leaves in contact with the fungus turned brownish and bleached. The medium further inhibited rooting of the explant, thus transforming the mutualistic fungus to an antagonistic one [16].

Many mechanistic details were elucidated in the model plant, *Arabidopsis thaliana*, during the course of its interaction with *P. indica* [6]. Apparently, *P. indica - A. thaliana* interaction represents a novel type of beneficial fungus-root interaction with early protein alterations in the plasma membrane and the endoplasmic reticulum [2, 4]. In *A. thaliana*, pronounced plant growth promotion and enhanced seed production were noticed in the presence of *P. indica*, involving synthesis of a leucine-rich protein repeat [17, 18], increased expression of nitrate reductase and a starch-degrading enzyme [18] as well as expression of a ß-glucosidase in the endoplasmic reticulum [19]. In addition, changes in levels of auxins and cytokinins were noticed [20].

Co-inoculation with *P. indica* increased the drought tolerance levels in *Arabidopsis* [21] and *Hordeum vulgare* [22]. *P. indica* increased the general stress tolerance of plants by elevating the levels of antioxidative enzymes [23]. The increase in growth performance induced by *P. indica* and *Sebacina vermifera* in *Nicotiana attenuata* was shown to be at the expense of herbivore resistance [24]. *P. indica* also increased the systemic resistance of the hosts against pathogenic fungal attack [22, 25]. An overview of the salient characteristic features is given in Fig. 1.

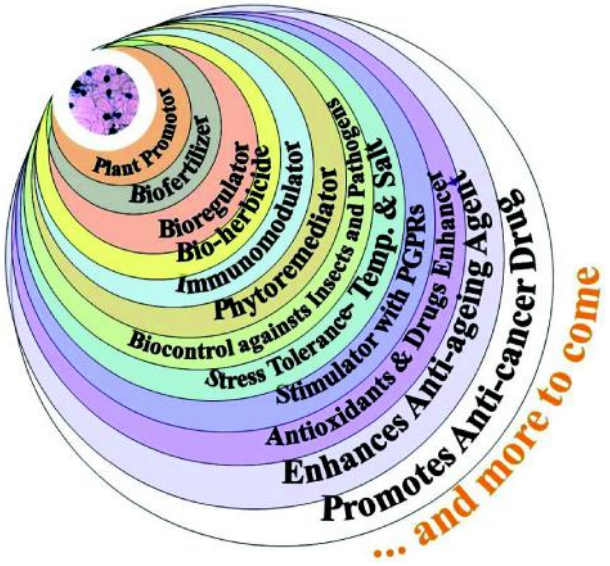


Fig. 1: Biotechnological applications of *Piriformospora indica*

Mass Propagation

The ease in the propagation of *P. indica* as axenic cultures makes its application in horticulture, floriculture and agriculture economically and practically feasible. It acts as a potent biofertilizer and biocontrol agent. This fungus can grow axenically on different synthetic media, the best being Hill and Käfer medium [1, 12, 26, 27] (Fig. 2a). This medium was used for the submerged cultivation of *P. indica* in 500 ml Erlenmeyer flasks. The effect of various culture parameters (inoculum size, agitation speed, working volume, initial pH and temperature) on the growth and sporulation of *P. indica* were studied by changing one parameter at a time while keeping others constant. With optimized culture parameters (inoculum size: 5%; agitation speed: 200 rpm; working volume: 20%; initial pH: 6.5; temperature: 30°C), the maximum dry cell weight of 13.47 g/l was obtained after 5 days in 500 ml Erlenmeyer flask with a volumetric productivity of 2.69 g L^{-1} d^{-1}[10]. The sporulation started after 6 days and the maximum spore yield of 7.46 x 10^7 spores/ml was obtained after 8 days of inoculation. All these values were much higher than the values observed with non-optimized Hill-Käfer medium.

In order to find a suitable carbon and nitrogen source for growth and sporulation, the fungus was cultivated on different carbon sources (glucose, fructose, sucrose, maltose, soluble starch, glycerol, cellulose and xylan) and different nitrogen sources ($NaNO_3$, $NaNO_2$, NH_4Cl, urea, NH_4NO_3, peptone, yeast extract and casamino acid hydrolysate) while all the other media components were kept at constant

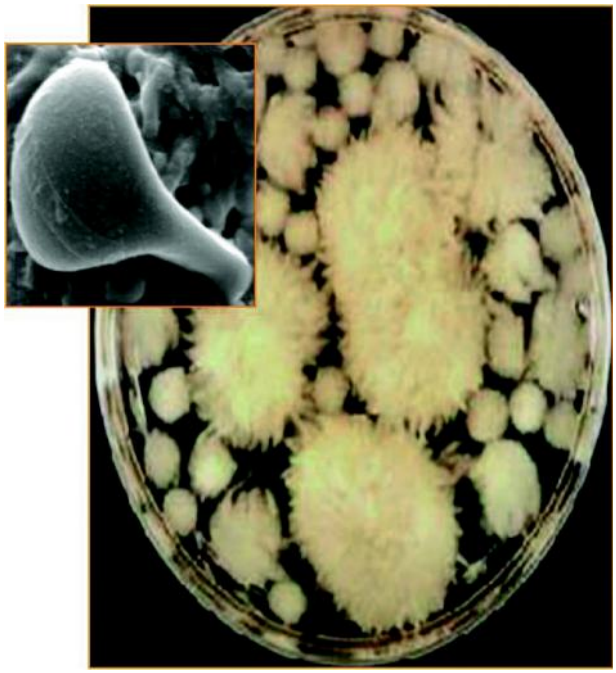


Fig. 2(A): Growth of *P. indica* on Hill- Kaefer broth (2001) under constant shaking condition at 25C for 7 days. Colonies of different developmental stages are shown. Mature colonies have the appearance of sea urchins

levels. The fungus could metabolize a number of carbon sources, but glucose and soluble starch were the best for growth and sporulation. The maximum growth and sporulation were recorded on the nitrogenous sources, peptone and yeast extract.

After the preliminary screening of components of Hill-Käfer medium by one-variable-at-a-time approach, the components which had substantial influence on the growth of *P. indica* were found to be peptone, yeast extract, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$. These four components were optimized using response surface methodology including central composite design (CCD). The optimum combination of the components was as follows: 3.0 g/l peptone, 3.0 g/l yeast extract, 1.83 g/l KH_2PO_4 and 0.65 g/l $MgSO_4 \cdot 7H_2O$. The concentrations of other components were the same as in the original Hill-Käfer medium without $NaNO_3$ and KCl. The glucose concentration was 20 g/l [10].

Of late, we have been successful in growing the fungus on Jaggery (Gur) prepared from sugarcane extract (*Saccharum officinarum*) wherein optimum chlamydospore production was obtained with 4% (w/v) of jaggery (Fig. 2b). The biomass had maintained the plant growth promoting properties. Chemical composition (in %) of Jaggery is: sucrose (60-85), glucose and fructose (5-15), protein (0.4), fat (0.05), minerals (0.6-1.0), calcium (0.4), magnesium and phosphorus (0.045), and iron (11). In comparison to Hill-Kaefer medium which yielded 12-15 g/l of the fungal biomass, the medium containing Jaggery (4 % w/v) yielded 16-18 g/l of the fungal biomass (Patent No. 944/DEL/2012).

In a scale up study, a 14 L bioreactor (Chemap AG, Switzerland) was used to grow *P. indica* in the optimized Hill- Käfer medium (as described above) and it supported maximum biomass and spore production [10]. Jaggery, which is economically feasible, needs to be optimized for higher biomass production of *P. indica* at a fermentor scale.

Plant Symbiotic Promotional Features

Role of Fungal Biomass

The inoculation of *P. indica* with the plant roots

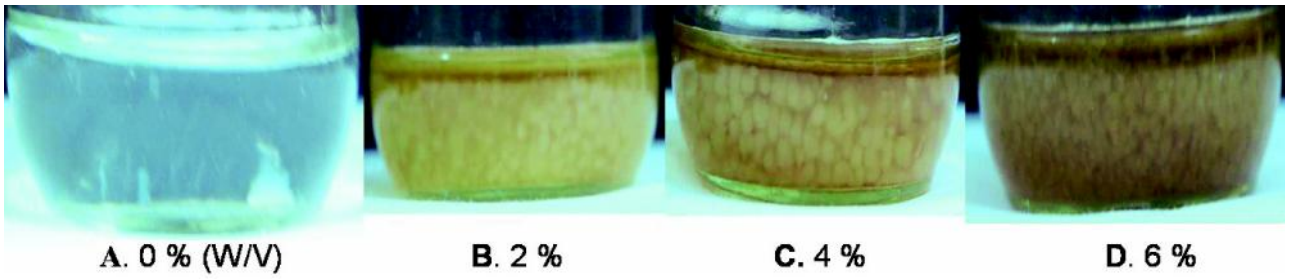


Fig. 2(B): Growth of *P. indica* in different concentrations of jaggery as nutrient. A. 0 % jaggery; B. 2 % jaggery; C. 4 % jaggery; D. 6 % jaggery

tremendously improves the growth and overall biomass production of diverse hosts [1-4, 6, 7, 9, 12, 15-22, 28-33]. Around 150 plant species of agricultural, horticultural and medicinal importance have been reported to interact with *P. indica* [6, 7, 15, 29-31, 33-40]. *Oryza sativa, Zea mays, Tridax procumbans* and *Brassica oleracea* var. *capitata* plants have shown early seed germination and increased seed production upon treatment with *P. indica* [36]. Increased biomass was observed in many plants, *viz*. *Oryza sativa, Zea mays, Phaseolus vulgaris, Tridax procumbans, Abrus precatorius, Solanum nigrum*, *Brassica oleracea* var *capitata*, *Brassica nigra, Nicotiana tabbacum, Saccharum officinarum, Lagenaria* spp. and *Spinacea oleracea* [5]. An increase in biomass as well as in the content of secondary metabolites was recorded in *Trigonella foenumgraecum*. Increase in chlorophyll content of plants treated with *P. indica* was also observed [41]. *P. indica*-colonized roots showed an early development compared to the control plants in all initial stages of growth, as suggested by early expression of developmentally regulated genes [22]. Sahay *et al*. [42] and Singh [43] observed that maize plants exhibited enhanced growth upon inoculation with *P. indica* and the fungus was able to colonize the root cortex. This fungus also promotes the growth of several tropical legumes tested (*Cicer arietinum*, *Phaseolus aureus*, *P. mungo*, *Pisum sativum* and *Glycine max*). Some of the plants of agricultural importance that interact positively with *P. indica* are shown in Fig. 3.

Artemisia annua, Bacopa monniera, Abrus precatorius, Stevia rebaudiana, *Linum album*, *Trigonella* spp., *Coleus forskohlii, Spilanthes* spp., *Withania* spp., *Chlorophytum* spp. and *Curcuma longa* are some of the important medicinal plants which have been reported to interact beneficially with the fungus (Fig. 4). The secondary metabolite content

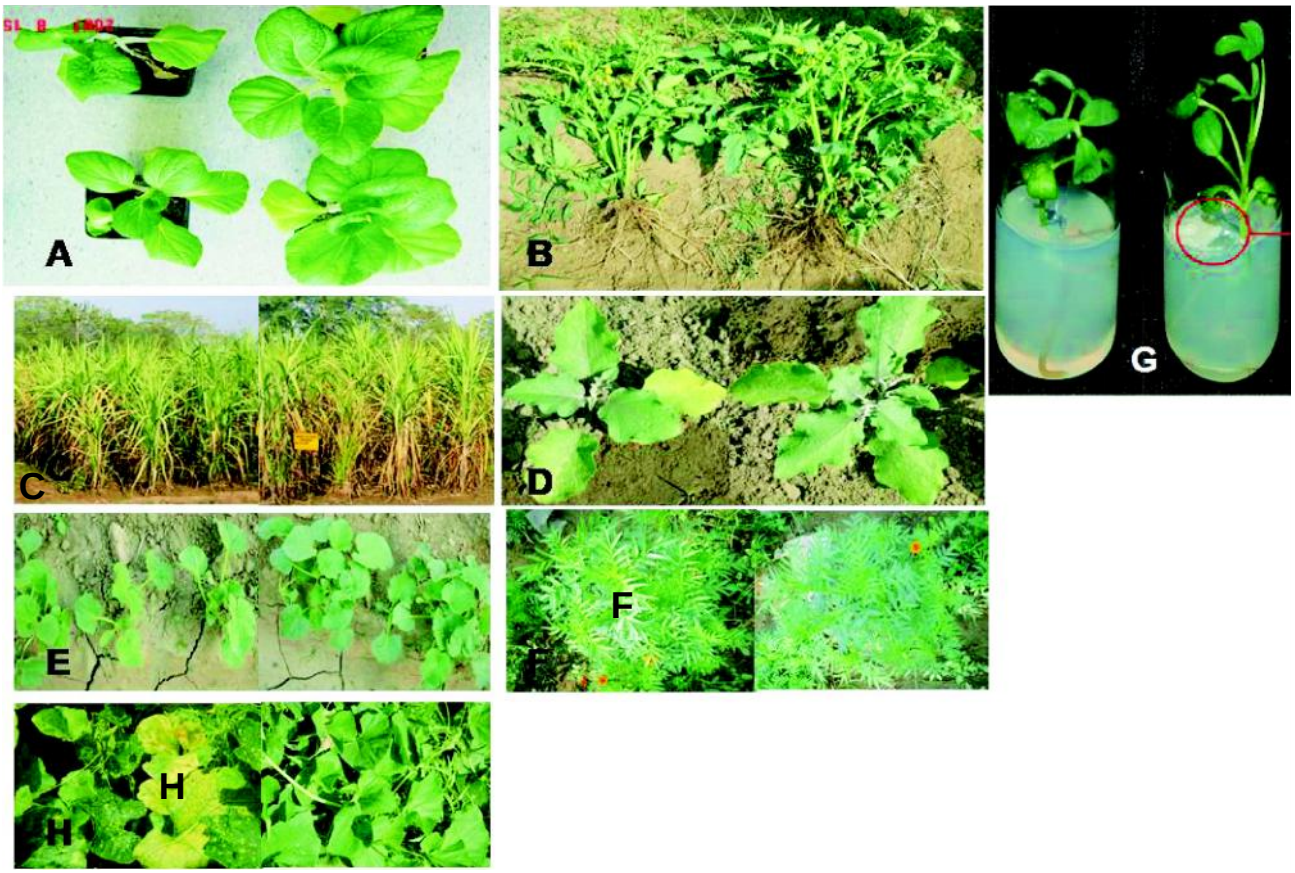


Fig. 3: ***P. indica*** **promotes plant growth. A.** ***Nicotiana tabaccum*****; B.** ***Lycopersicon esculentum*****; C.** ***Saccharum officinarum*****; D.** ***Solanum melongena*****; E.** ***Citrullus lanatus*** **(water melon); F.** ***Tegetus erectus*****; G.** ***Trigonella*** **sp.; H.** ***Lagenaria*** **sp. (bottle gourd). Left: control; Right: fungus treated**

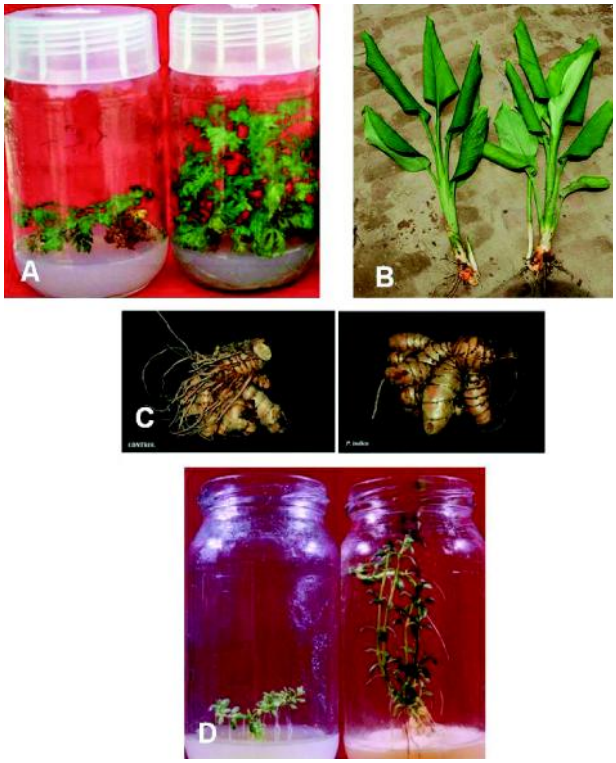


Fig. 4: ***P. indica*** **promotes growth of medicinally important plants (*In vitro* experiments). A. *Artemisia annua*; B. *Curcuma longa*; C. Rhizome size of *Curcuma longa* D. *Bacopa monniera*. (left: control; right: *P. indica* treated plants)**

of all these medicinal plants increased several folds (e.g. Artimisinin-2.6 %, Bacoside-3.5 %, Curcumin-19 %) upon interaction with *P. indica.* Reduction in growth of hairy roots of the medicinal plant *Linum album* was observed upon co-cultivation of live fungal cells with hairy roots. In spite of a reduction in hairy root biomass, an increase in lignin content was observed. The hairy root cultures co-cultivated with 1 to 5 g/l of fungal biomass at day 10, 11, 12 and 13 depicted a higher podophyllotoxin (PT) and 6-methoxypodophyllotxin (6-MPT) content in the roots in comparison to the fungus-free control culture. The highest increase in PT (8.48 mg/g) and 6-MPT content (3.78 mg/g) were obtained with a fungal concentration of 2.0 g/l added to growing hairy root cultures of *L. album* (Iran cultivar) on 12th day, i.e., for 48 h. Promotion of early flowering was observed in *Coleus forskohlii*, *Nicotiana tabaccum*, *Chlorophytum* spp., *Spilanthes calva* and most recently in *Brassica napus* (Fig. 5).

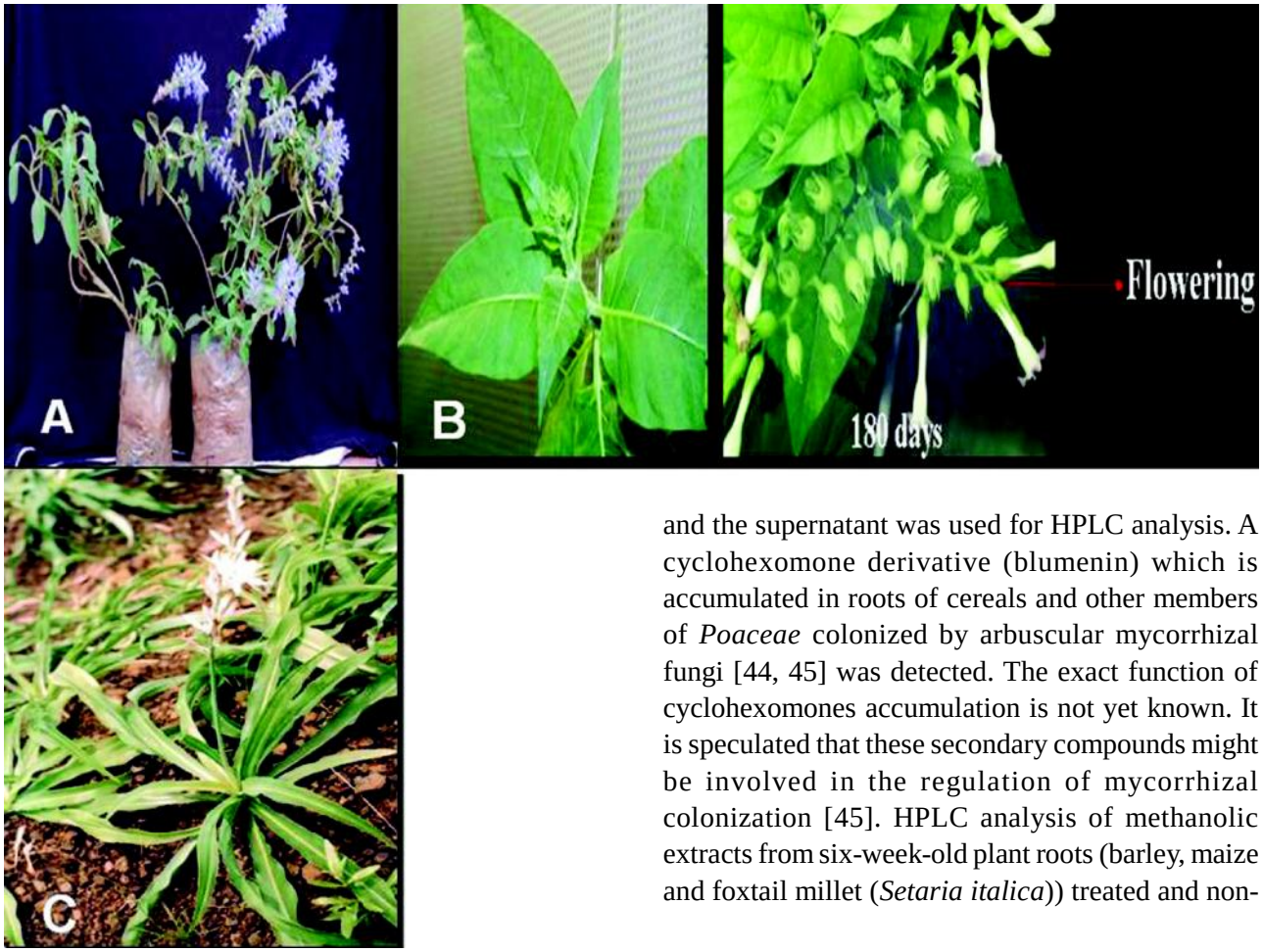
Role of Fungal Culture Filtrate

Not only the mycelium but also the culture filtrate of the mycelium contains fungal exudates, minerals, hormones, enzymes, proteins, etc. [9]. It has been shown by *in vitro* experimentation that even a very small amount (50 uL) of culture filtrate is sufficient to promote root and shoot growth (Fig. 6). To prove the effect of culture filtrate, pot-culture experiments were done. 15-day-old seedlings (brinjal, broccoli, beans, sunflower, cabbage, maize, *Bacopa* and tobacco) were transferred to disposable plastic pots containing vermiculite (autoclaved) and sand (acid washed) in the ratio of 3:1. An amount of 15 ml freshly eluted *P. indica* culture filtrate was applied to each pot. Minimal Kalevar-Majumdar (KM) medium and equal amounts of double sterilized distilled water served as control. As in Sunflower, an increase in the root length (35.85 % over control), shoot length (19.86 % over control) and plant biomass (36.7 % over control) was observed in the *P. indica* treated plants.

Factors for Plant Stimulation

Aliquots of 1 g fresh weight of freshly harvested *P. indica* hyphae, washed with water, were prepared and then treated twice with 5 ml of 80 per cent aqueous methanol. This mixture was centrifuged at 6,000 g for 15 min and the supernatant was used for HPLC analysis. The analysis showed seven peaks in the hyphal extract and one main peak in the culture filtrate. A major peak in the preparative HPLC analysis of hyphal extract and culture filtrate was identified as benzoic acid. The function of this compound is not yet clear. However, compounds identical to benzoic acid and its analogues (benzoic acid, a-hydroxybenzoic acid, 3-4 di-hydroxybenzoic acid, vanillic acid, cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid) did not show any stimulation on the plants tested. As of now, the nature of the stimulatory factor which promotes the plant growth is not known [1].

Intact roots of different plants were harvested and washed with water. Aliquots of 1 g were treated twice with 5 ml of 80 per cent aqueous methanol. This mixture was centrifuged at 6,000 g for 15 min



and the supernatant was used for HPLC analysis. A cyclohexomone derivative (blumenin) which is accumulated in roots of cereals and other members of *Poaceae* colonized by arbuscular mycorrhizal fungi [44, 45] was detected. The exact function of cyclohexomones accumulation is not yet known. It is speculated that these secondary compounds might be involved in the regulation of mycorrhizal colonization [45]. HPLC analysis of methanolic extracts from six-week-old plant roots (barley, maize and foxtail millet (*Setaria italica*)) treated and non-

Fig. 5: ***P. indica*** **induces early flowering. A.** ***C. forskohlii*****; B.** ***N. tabaccum*****; C.** ***Chlorophytum*** **spp. (left: control; right:** ***P. indica*** **treated plants)**

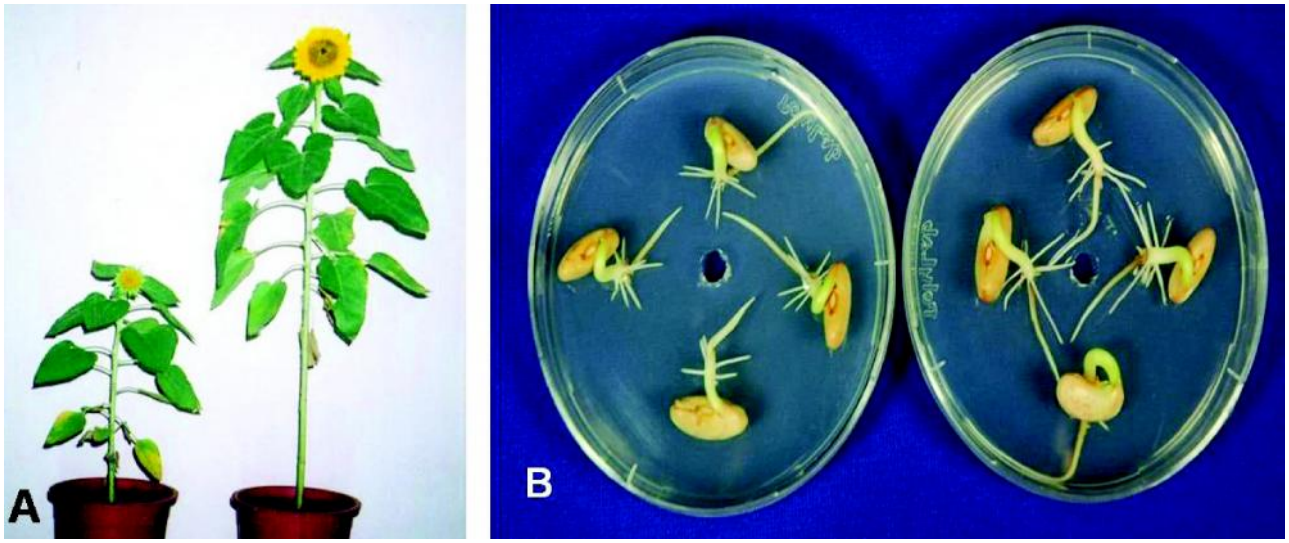


Fig. 6: ***P. indica*** **culture filtrate promotes plant growth and seed germination. A.** ***Helianthus annus*****; B.** ***Phaseolus vulgaris*** **(left: control; right:** ***P. indica*** **treated plants) (Comment: the images of Petri dishes in B appear distorted as if the image has been elongated in one dimension – original unstretched image is required Need ful has been done and attached in jpeg version)**

treated with *P. indica* showed quantitative but no qualitative changes. There were no changes recorded in case of rice and wheat. The UV spectra obtained from HPLC photodiode array detector showed a cluster of peaks between 7.5 and 12.5 min of retention time for extracts from maize, barely, rice and foxtail millet co-inoculated with *P. indica*, indicating the presence of indole-derivatives, e.g., tryptophane, tryosine and tyramine or their derivatives (*unpublished results*).

Root extracts of maize showed the presence of cyclic hydroxamine acids like DIMBOA (2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one) in wheat but not in rice, barley and *Setaria*. HPLC analysis of methanolic extracts of infected maize roots showed eight different peaks with a photodiode array detector with typical UV-spectra of benzoxazinone derivatives. None of these peaks was identical to DIMBOA or DIBOA. The chemical structure of these compounds needs to be identified.

Phosphate and Nitrate Transport

The interaction of host plant roots with *P. indica* is accompanied by an enormous uptake of nitrogen and phosphorous from the environment [18, 10]. Role of *P. indica* in P-acquisition by the root, especially in the arid and semi-arid regions has been suggested from the ^{32}P experiments. Cloning and functional analysis of a gene encoding for a phosphate transporter (*PiPT*) was reported from this root endophyte [10]. The *PiPT* polypeptide belongs to the Major Facilitator Superfamily (MFS) and exhibits 12 transmembrane helices divided into two halves that are connected by a large hydrophilic loop in the middle. The function of the protein encoded by *PiPT* was confirmed by complementation of a yeast phosphate transporter mutant. *PiPT* is actively involved in the phosphate transportation and in turn, *P. indica* helps to improve the nutritional status of the host plant [10]. Recently, Pedersen *et al*. [46] reported the crystal structure of a high affinity phosphate transporter, PiPT from *P. indica*, which indicates both proton and phosphate exit pathways and suggested a modified asymmetrical 'rocker switch' mechanism of phosphate transport.

In *Arabidopsis* and tobacco seedlings, the growth promotional activity of the fungus is attributed to an increase in nitrate uptake, and an up-regulation of nitrate reductase (Nia2) gene and the starch-degrading enzyme glucan-water dikinase (*SEX1*) in roots and shoots [18]. Increase in the activity of NADH-dependent nitrate reductase in the colonized roots resulted in a massive transfer of nitrogen into the aerial parts of the seedling.

Recent studies have also shown that sulphur metabolism is stimulated by the fungus. Genes that code for several plastid-localized enzymes required for sulphate reduction are up-regulated by *P. indica* in *Arabidopsis* roots and gene inactivation studies confirmed that they are required for the benefits to the plants (Unpublished data).

Resistance to Abiotic Stress

Drought Stress

P. indica efficiently confers plants with tolerance to abiotic stresses like drought tolerance in *Arabidopsis* and Chinese cabbage [21, 28, 31]. Fungal colonized *Arabidopsis* plants exposed to drought, showed increased drought tolerance with continuous growth while the growth was inhibited in untreated controls [21, 28]. Higher chlorophyll contents and increased photosynthetic efficiencies were recorded in the fungus-colonized plants under drought-stress when compared to uncolonized plants [21]. The genes involved in the diverse processes of drought stress tolerance, i.e., response to dehydration (RD)29A, early response to dehydration (ERD)1, phospholipase dδ (PLD), the transcription factor gene ANAC072, dehydration-response element binding protein (DREB)2A, salt and drought-induced ring finger (SDIR)1, calcineurin b-like protein (CBL)1 and cbl-interacting protein kinase (CIPK)3, were rapidly up-regulated in the leaves of *P. indica*–colonized *Arabidopsis* seedlings compared to the controls [21]. Up-regulation of the 2 ascorbate reductase genes *viz. MDAR2* and *DHAR5* was observed in the roots and shoots of colonized *Arabidopsis* seedlings exposed to drought [28]. Plant growth and seed production were not affected in the fungal colonized *mdar2* and

dhar5 mutants indicating that MDAR2 and DHAR5 are essential for maintaining sufficient ascorbate levels required for a balanced mutualistic state [28]. Thus indicating that *P. indica* confers drought tolerance to *Arabidopsis* by priming the aerial parts of the plant for an early and increased expression of drought tolerance-related genes [21, 28].

P. indica Influences Metabolism under Extremely Low Temperature

Interestingly, although *P. indica* was screened from hot desert of Rajasthan, India (+40 to +50ºC), it showed a positive influence on seed germination under extreme low temperatures (Leh-Ladakh, India; altitude: 3,300 meters; temp.: –30 to 4ºC) as studied in 12 leafy vegetable plants (Table 1). The seed germination was observed to be 100 % in case of cabbage, endive, swisschord (Palak), swisschord (Red), radish and onion within 25 days of sowing. In contrast, no seed germination was noticed in untreated control. Significant increase in growth of cabbage, cauliflower heads and beetroot bulbs was recorded in the fungus treated plants (Fig. 7). The salient features have been patented (Patent no. 709/DEL/ 2011 dated 15th March 2011).

Table 1: Influence of *Piriformospora indica* on seed germination under extreme low temperatures ranging from –30ºC to + 4ºC

Hosts	Days	No. of seeds germinated upon fungal treatment*
Cabbage	25	100
Endive	25	100
Swisschord (Palak)	25	100
Swisschord (Red)	25	100
Radish	25	100
Onion	25	100
Carrot	21	84
Cauliflower	21	84
Beetroot	20	80
Peas	15	60
Snowpea	12	48

*There was no germination of seeds up to 45 days in untreated controls. Total No. of seeds used for the treatment were 100

Nutrient and Salt Stress

P. indica imparted nutrient and salt resistance to the co-cultivated tomato plants (*Solanum lycopersicum* "Roma") by activating the antioxidant metabolism, which leads to the accumulation of ascorbate. The fruits of the *P. indica* inoculated plant maintained their lycopene content independent of the growth conditions. Gosal *et al*. [32] showed that biotization of micropropagated *Chlorophytum* sp. with this endophyte improves plantlet survival rate and the nutrient acquisition capacity. Uptake of Cu, Fe, Zn and Mn were improved in the plantlets inoculated with *P. indica.*

Co-inoculation of micropropagated sugarcane plantlets with *P. indica* improved their survival rate by 12% upon their transfer to soil (Table 2). In the cultivar CoJ 83, the root colonization was 91.8% and in cultivar CoJ 88 it was 92.5% after four weeks of growth in greenhouse. Cane yield and yield components (tillering and cane height) in bio-hardened variety of CoJ 88 were significantly higher than that of both non-inoculated micropropagated and non-inoculated conventionally propagated sugarcane. Similar results were obtained in the ratoon crop.

Deficiency of the mineral element iron was observed in the un-inoculated ratoon crop plants, whereas the uptake of iron and copper was promoted in the fungus inoculated plants (Table 3). However, a decrease in the uptake of zinc was observed, which needs further elucidation.

Cadmium (Cd) pollution in soil poses a serious threat to rice quality. Thus, it is important to find ways for increasing the tolerance of rice to Cd and also to limit Cd accumulation. In order to develop Cd tolerant plants, rice plants were exposed to heavy metals in presence of cell wall extract of *P. indica.* It was found that the cell wall extract of *P. indica* increased tolerance of rice to cadmium stress (Unpublished work).

Fig. 7: *P. indica* promotes plant growth under extreme temperature stress in the cold deserts of Laddakh. (Beetroot: left-control; right-*P. indica* treated plants)

Table 2: Interaction of *Piriformospora indica* with *Saccharum officinarum* (var.CoJ88)- Field trial Experiments were performed at Punjab Agricultural University, Ludhiana (courtesy- Dr. Gosal)

Treatments	Tiller number/ clump	Cane number/ clump	Cane height (cm)	Cane girth (cm)	Sugar content (in Brix)	Weight/ clump (kg)	Weight/ plot (kg)
Control	9.27	8.10	179	2.22	18.35	6.50	122.2
P. indica	17.2	15.90	191	2.21	21.40	7.34	138.3
CD (5%)	2.59	2.51	NS	NS	1.99	NS	2.39

Table 3: Availability of mineral nutrients in *Piriformospora indica* treated soils [Field trial Experiments were performed at Punjab Agricultural University, Ludhiana (courtesy- Dr. Gosal)]

Treatments	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	K (%)	P (%)	S (%)
Control	202.2	25.0	4.9	1.87	0.24	0.086	0.095
P. indica	281.4	30.2	10.8	1.31	0.40	0.088	0.092
CD (5%)	47.07	NS	0.88	0.33	NS	NS	NS

Interaction with Rhizobacteria

An increasing demand for improving the sustainability of low-input agriculture has resulted in greater interest in rhizosphere microorganisms that increase soil fertility or improve plant nutrition and health [47]. The biocontrol capabilities of fluorescent *Pseudomonas* spp., *Serratia* spp., *Bacillus* spp. and *Burkholderia* spp. result largely from their ability to produce a battery of antifungal metabolites, which can also affect beneficial fungal-root symbioses [48]. In this communication, we report the intense interaction between *P. indica* and economically important rhizobacteria. It was observed that while some rhizobacteria could promote growth and root colonization of the fungus or behave neutral in the interaction with *P. indica*, others severely inhibited its development. While designing the microbial consortium for imparting betterment to the crop

productivity, one has to evaluate the compatibility interactions between microorganisms.

Interaction of Rhizobacteria with P. indica

The co-inoculation of rhizobacteria along with *P. indica* influenced the stimulatory effect of *P. indica* in the gnotobiotic barley system. *Azospirillum brasilense* Sp245 had no effect, *Serratia liquefaciens* MG1 inhibited the root growth stimulating property of *P. indica*, and interestingly *Pseudomonas putida* IsoF enhanced the root growth stimulation property of *P. indica* (Fig. 8A). FISH-analysis using probes specific for the bacteria and fungi and confocal laser scanning microscopy demonstrated a close physical interactions between *Pseudomonas putida* IsoF and *P. indica* (Fig. 8B) [8], while in the case of inhibitory interactions, any close contact was rare (data not shown).

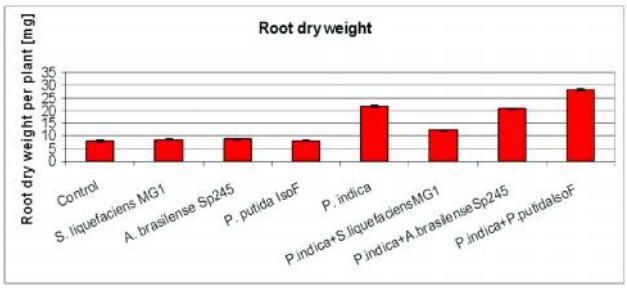


Fig. 8(A): Root dry weight of axenically grown barley seedlings under the influence of *P. indica* and the rhizobacteria *A. brasilense* Sp245, *Serratia liquefaciens* MG1 and *Ps. putida* IsoF (control: not inoculated)

Structural Changes of P. indica *Induced by Inhibitory P. fluorescens WS5*

P. fluorescens WS5 and *Burkholderia cepacia* LA3 [49] were inhibitory to the growth of the fungus as depicted in the *in vitro* inhibition assays on solid agar medium (Fig. 9A-C). In controls without rhizobacteria, the mycelial mat was dense with thin and densely interwoven hyphae of 0.5-2 µm in diameter. In the presence of *P. fluorescens* WS5, no observable mycelial mat was produced, the hyphae were thin and lysis of some of the hyphae was observed.

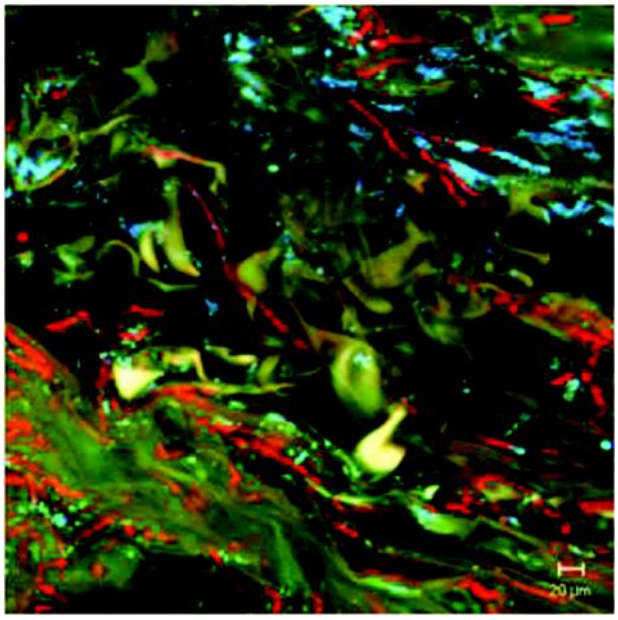


Fig. 8(B): Colonization of barley roots in an axenic system by *P. indica* and *Pseudomonas putida* IsoF. FISH-analysis and confocal laser scanning microscopy (LSM510, Zeiss Jena, Germany) were performed. The fluorescence labeled oligonucleotide probes Eub339 I, II, III – Cy5 (blue in rgb-image) for bacteria (*Ps. putida* IsoF) and EuK-Cy3 (red in the rgb-image) for the fungus *P. indica* were applied

The inhibitory metabolites diffused from the *B. cepacia* LA3 exerted a complex influence on the metabolomic pathways of *P. indica*. For further study, saponin was selected as a model component mixture, which is produced by plants and is a complex mixture of biomolecules like steroids and terpenes with surfactant and inhibitory activities. Also in *Bacillus* and *Pseudomonas*, cyclic lipopeptides are known to be potent inhibitors of pathogenic fungi [48]. A concentration dependent growth inhibition of *P. indica* was observed in a medium containing saponin (Fig. 10). At a concentration of 0.1 and 0.5% of saponin, the fungus grew slowly and at 1%, the growth was completely blocked [50].

Two contrasting observations, i.e. differential response of *P. indica* to the presence of rhizobacteria and its ability to survive in soil and colonize plant roots, led to the hypothesis that microbial communities interact through diffusible metabolites to counteract the inhibitory or stimulatory factor(s), thereby maintaining the delicate balance between

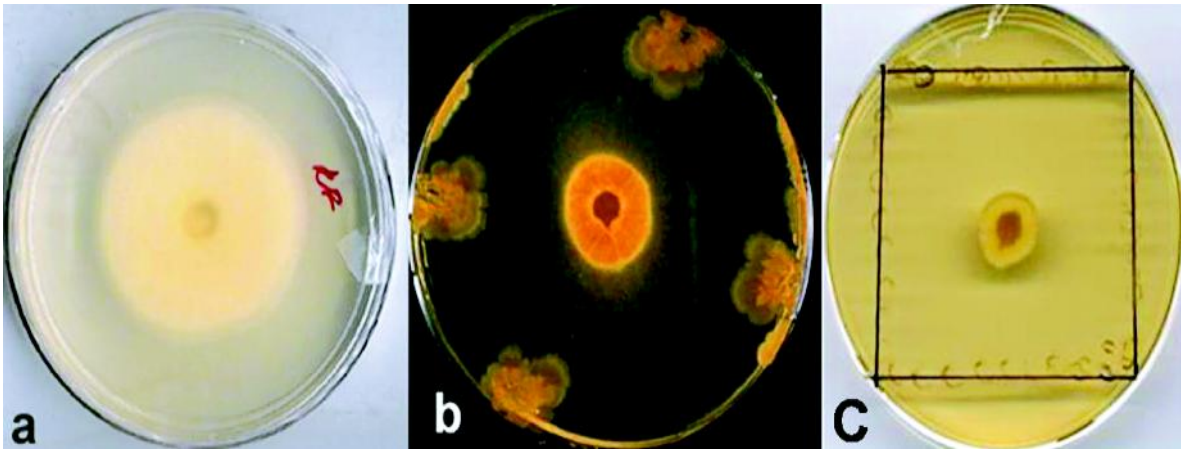


Fig. 9: ***P. indica*** **vs** ***Pseudomonas putida*** **IsoF and** ***Burkholderia cepacia*** **LA3 incubated on modified aspergillus medium (A) control:** ***P. indica*** **alone (B) Interaction of** ***P. indica*** **(placed at the center) with** ***Pseudomonas putida*** **IsoF (on all four corners) (C) Interaction of** ***P. indica*** **(placed at the center) with** ***Burkholderia cepacia*** **LA3 (on all four sides)**

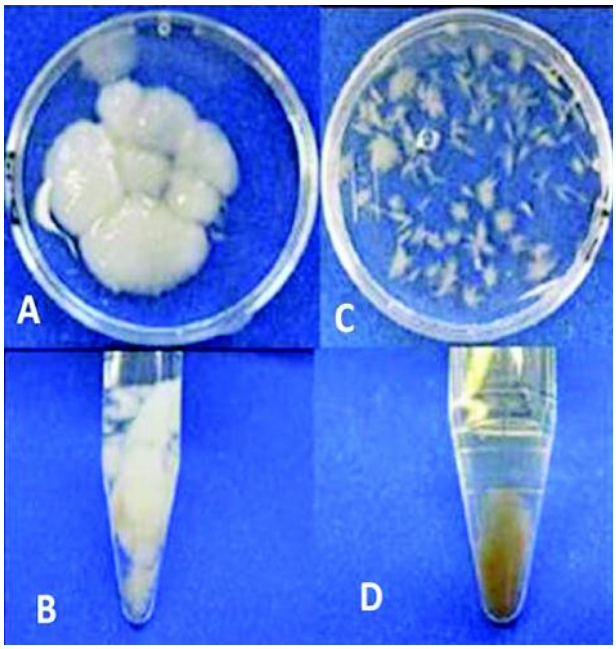


Fig. 10: ***P. indica*** **grown in (A) agar plate and (B) liquid medium and the fungus grown in presence of saponin at a concentration of 0.5% in (C) agar plate and (D) liquid medium. Inhibition in the growth was observed by the presence of saponin at the applied concentrations**

diverse soil microorganisms. To prove this concept, *in vitro* assays using axenic barley seedlings were carried out using the widely distributed PGPR *Azospirillum brasilense* Sp245, *Pseudomonas putida* IsoF and *Serratia liquefaciens* MG1. The results demonstrated a wide range of possible interactions – from inhibition to stimulation – taking place on the root surface. The possible molecular mechanisms of stimulatory interactions could be manifold, but these are not the focus of this communication. Concerning the inhibitory effects, antibiotics and lipopeptides produced and excreted by many biocontrol active rhizobacteria are responsible for the inhibition of *P. indica* by the plant growth enhancing inoculant *B. amyloliquefaciens* FZB42 and the biocontrol rhizobacterium *P. fluorescens* SS101. This inhibition of *P. indica* by diffusible bacterial metabolites of *P. fluorescens* WS5 and *Burkholderia cepacia* LA3 is demonstrated in more morphological and metabolomic details.

To investigate if similar mixture has identical influence on the growth of the *P. indica*, saponin was used as a model component since it is a complex mixture of secondary metabolites mostly of plant origin. The saponin treatment at amounts of 0.1-1%, suppressed the growth of the fungus, which appears to be not reported earlier. In contrast to the influence with diffusible inhibitory metabolites of certain rhizobacteria, the ubiquinone biosynthesis as well as limonene and pinin degradation pathways were not affected by saponin.

The diverse mycorrhiza-like qualities present in axenically cultivable *P. indica* is unique [51]. The studies may shed light on the ecology and evolution of a fascinating group of fungi whose striking

biodiversity and ecological importance has only been recently started to be recognized [6, 28, 52]. *P. indica* has been documented to benefit plant growth and increase resistance against pathogens in a broad range of host plants [4]. This study has special significance as the fungus is being exploited for biotechnological applications in the area of agriculture, forestry, arboriculture, flori-horticulture in field, and hydroponic cultivation of several vegetable and aromatic hosts. For a possible combined application of *P. indica* with plant growth promoting rhizobacteria, there is a need to establish that these rhizobacteria are not inhibitory to *P. indica*.

Future Prospective and Parting Questions

The effect of bacteria on mycorrhiza, as described in the concept of mycorrhizal helper bacteria [53], has not been documented yet in this particular mycorrhiza-like symbiotic system of *P. indica* interacting with a wide variety of plants with high practical importance. A tightly associated endofungal bacterium, *Rhizobium radiobacter*, has been identified *in situ* and localized using fluorescence *in situ* hybridization and confocal laser scanning microscopy in hyphae of *P. indica* cultures [54]. However, the functional role of this bacterium with *P. indica* and its interaction with plant roots is not known yet. This bacterium can be grown in pure culture outside the fungus, *P. indica* could not be cured yet from this bacterium. Other members of the Sebacinales have other endofungal bacteria, like *Paenibacillus* spp., in *S. vermifera.* However, this bacterium was not yet successfully grown in pure culture without the fungus [54].

The versatile nature of this fungus has been thoroughly reviewed [55, 56] but the exact mechanism of plant growth promotion and the trigger for early flowering in the plants induced by *P. indica* needs to be further studied. In addition, the culture filtrate of the fungus needs to be completely characterized in order to pin point the active ingredients involved in the plant growth promotion. The powder formulation of the fungus is being commercialized under the trade name 'ROOTONIC' [57] (www.amity.edu/aims/ResearchandPublication.asp.

Acknowledgements

The authors are thankful to the Indian Council of Agricultural Research, Department of Biotechnology and Defence Research and Development Organsation for partial financial support.

References

1. Varma A, Singh A and Sudha M *et al*. (2001) *Mycota 9* Springer-Verlag Germany pp 125-150
2. Peškan-Berghoefer T, Shahollari B, Giong PH, Hehl S and Markert C *Physiol Plant* **122** (2004) 465-477
3. Pham GH, Kumari R, Singh AN *et al*. (2004) *Plant Surface Microbiology,* Springer-Verlag, Germany pp 593-616
4. Shahollari B, Varma A and Oelmuller R *J Plant Physiol* **162** (2005) 945-958
5. Prasad RP (2008) *Studies on Interaction Between Symbiotic Fungus (Piriformospora indica), Rhizobacteria and Selected Plants*, Ph D Thesis Merrut University, India
6. Oelmuller R, Sherameti I, Tripathi S *et al*. *Symbiosis* **49** (2009) 1-17
7. Fakhro A, Andrade-Linares DR, von Bargen S *et al*. *Mycorrhiza* **20** (2009)191-200
8. Varma A, Bakshi M, Lou B *et al*. *Agri Res* **1** (2012) 117-131
9. Verma S, Varma A, Rexer K-H *et al*. *Mycologia* **90** (1998) 896-903
10. Yadav V, Kumar M, Deep DK *et al*. *J Biol Chem* **285** (2010) 26532-26544
11. Varma A, Sherameti I, Tripathi S *et al*. (2012) *The Mycota 9: Fungal Associations* Springer-Verlag Germany pp 231-254
12. Varma A, Verma S, Sahay NS *et al*. *Appl mol microbiol* **65** (1999) 2741-2744
13. Weiss M, Selosse MA, Rexer K-H *et al*. *Mycological Res* **108** (2004) 1003-1010
14. Weiss M, Sykorova Z, Garnica S *et al*. *PloS ONE* **6** (2011) e16793
15. Sahay NS and Varma A *FEMS Microbiol Lett* **181** (1999) 297-302
16. Kaldorf M, Koch B, Rexer K-H, Kost G and Varma A *Plant Biol* **7** (2005) 210-218

17. Shahollari B, Vadassery J, Varma A and Oelmuller R *Plant J* **50** (2007) 1-13
18. Sherameti I, Shahollari B, Venus Y *et al*. *J Biol Chem* **280** (2005) 26241-26247
19. Sherameti I, Venus Y, Drzewiecki C *et al*. *Plant J* **54** (2008b) 428-439
20. Vadassery J, Ritter C, Venus Y *et al*. *Mol Plant Microbe* **21** (2008) 1371-1383
21. Sherameti I, Tripathi S, Varma A *et al*. *Mol Plant Microbe* **21** (2008) 799-807
22. Waller F, Achatz B, Baltruschat H *et al*. *Proc Nat Acad Sci* (USA) **102** (2005) 13386-13391
23. Vadassery J, Ranf S, Drzewiecki C *et al*. *Plant J* **59** (2009) 193-206
24. Barazani O, Benderoth M, Groten K *et al*. *Oecologia* **146** (2005) 234-243
25. Serfling A, Wirsel SGR, Lind V *et al*. *Phytopathology* **97** (2007) 523-531
26. Hill TW and Kaefer E *Fungal Genet News Lett* **48** (2001) 20-21
27. Pham HG, Kumari R, Singh A *et al*. (2004) *Plant Surface Microbiology*, Springer-Verlag, Germany, pp 593-613
28. Vadassery J, Tripathi S, Prasad R *et al*. *J Plant Physiol* **166** (2009b) 1263-1274
29. Kumar M, Yadav V, Tuteja N *et al*. *Microbiology* **155** (2009) 780-790
30. Achatz B, Rüden SV, Andrade D *et al*. *Plant and Soil* **33** (2010) 59-70
31. Sun C, Johnson JM, Cai D *et al*. *J Plant Physiol* **167** (2010) 1009-1017
32. Gosal SK, Karlupia A, Gosal SS *et al*. *Indian J of Biotech* **9** (2010) 289-297
33. Singh AR, Singh AN and Varma A *Indian J of Biotech* **1** (2002) 372-376
34. Rai MK, Acharya D, Varma A, *et al*. (2001) *National Workshop on Conservation of Medicinal Aromatic Plants* CFHRD, Chhindwara on December 26-27, pp105-110
35. Singh AN, Singh AR, Kumari M *et al*. (2003) *Biotechnology in Sustainable Biodiversity and Food Security,* Oxford & IBH, pp 101-124
36. Kumari R, Sachdev M, Garg AP *et al*. *Natural Product Radiance CSIR* **3** (2004) 396-400
37. Kumari R, Pham GH, Prasad R *et al*. (2004) *A Basic Research and Applications: Mycorrhizae,* IK International-India, New York and Kluwer academic Press, Holland, pp 259-295
38. Kumari R, Sachdev M, Garg AP *et al*. (2004) *A Basic Research and Applications: Mycorrhizae,* IK International-India, New York and Kluwer academic Press, Holland, pp 339-362
39. Rai M and Varma A *Electronic Journal of Biotechnology* **8** (2005) 107-112
40. Chauhan AK, Das A, Kharkwal AC *et al*. (2006) *Microbiology Series-Microbes: Health and Environment,* I K International Publishing House Pvt. Ltd., New Delhi, pp 1-12
41. Sharma M, Chauhan G, Chandra A *et al*. *Intl J Phytomed Related Inds* (2011) (Accepted)
42. Sahay NS, Singh A and Varma A *Indian J Exp Biol* **36** (1998) 1069-1086
43. Singh A (2004) *Immuno-Characterization of* Piriformospora indica *and Other Identical root Endophytes,* Ph D Thesis, School of Life sciences, Jawaharlal Nehru University, New Delhi
44. Maier W, Peipp H, Schmidt J *et al*. *Plant Physiol* **109** (1995) 465-470
45. Fester T, Maier W and Strack D *Mycorrhiza* **8** (1998) 241-246
46. Pedersen BP, Kumar H, Waight AB *et al*. *Nature* (2013) Doi: 10.1038/nature12042
47. Ryan PR, Dessaux Y, Thomashow LS *et al*. *Plant and Soil* **321** (2009) 363-383
48. Raajimakers JM, Paulitz TC, Steinberg C *et al*. *Plant and Soil* **321** (2009) 341-361
49. Jha B, Thakur MC, Gontia I *et al*. *Eur J Soil Biol* **45** (2009) 62-72
50. Varma A, Fekete A, Srivastava A *et al*. (2013) Piriformospora indica: *Sebacinales and Their Biotechnological Applications,* Springer-Verlag, Germany, pp 201-222
51. Aguilaniu H, Durieux J and Dillin A *Genes Development* **19** (2005) 2399-2406
52. Franken P *Appl Microbiol Biotechnol* **96** (2012) 1455-1464
53. Garbaye Tansley J *New Phytol* **128** (1994) 197-200
54. Sharma M, Schmid M, Rothballer M *et al*. *Cell Microbiol* **10** (2008) 2235-2246
55. Varma A, Kost G and Oelmueller R (2013) Piriformospora indica: *Sebacinales and Their Biotechnological Applications*, Springer-Verlag, Germany
56. Unnikumar KR, Sree KS and Varma A *Symbiosis* **60** (2013) 107-113
57. Varma A, Bajaj R, Agarwal A *et al*. (2013) *Memoirs of Rootonic- the magic Fungus: Promotes agriculture, Horticulture and Forest productivity*, Amity University Press, Noida, India.