Plant productivity determinants beyond minerals, water and light: *Piriformospora indica* – A revolutionary plant growth promoting fungus

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Biotic factors, along with the more obvious abiotic factors, determine and greatly influence the productivity and health of the plants. Arbuscular mycorrhizal fungi, AMF, the root-interacting predominant microbiota play an indispensable role in upgrading plant growth vigour and survival. However, the non-availability of the axenic culture is a great bottleneck for the fundamental studies and their biotechnological applications. *Piriformospora indica* is a newly described axenically cultivable phytopromotional endosymbiont, which mimics the capabilities of AMF. The fungus having a broad host spectrum shows pronounced growth-promotional effects. It mobilizes the insoluble phosphates and translocates the phosphorus to the host in an energy-dependent process. As a biological hardening agent of micropropagated plants, it renders more than ninety per cent survival rate for laboratory to field transferred plantlets. The successful isolation of regenerative protoplasts of *P. indica* opens up important possibility of improving symbiosis by transgenic manipulation of fungal component in a symbiosis-specific manner. The immobilization of the fungus stabilizes the infective capacity of the fungus and promises its use as a viable inoculum for biotechnological applications and long-distance transportation. The axenic cultivability of *P. indica* on economically viable synthetic media makes it suitable for mass scale inoculum production for application in agro-forestry and horticulture. In sum up, solar energy, water and soil nutrients are undoubtedly essential for plant productivity but the interaction with useful and friendly microbes also exert a tremendous impact.

IT is a general belief that plants, because they are autotrophs, can carry out all the functions of life with the availability of the so-called abiotic factors such as solar energy, moisture and mineral nutrients. However, what is not generally realized is that the plants, as all living organisms, also interact with the biotic factors, and their underground root system is under the direct influence of a diverse group of micro-organisms[1]. The mycorrhizal fungi and PGPRs (plant growth promoting rhizobacteria) being mutualistic symbionts, control, in many ways, the plant health[2]. More than 90% of the terrestrial plants (angiosperms, gymnosperms, pteridophytes, bryophytes and some algae) are colonized by mycorrhizal fungi. Arbuscular mycorrhizal fungi (AMF), which belong to the order Glomales (Zygomycota), are an integral part of the living plant system in a great majority of terrestrial plants. They penetrate into the root cortex of the host and differentiate into special intracellular structures called arbuscules. These fungi play a pivotal role in plant health, especially in stressed soils which are not normally fertilized and are dependent on rain-fed irrigation[3]. These fungi play an indispensable role in upgrading plant growth, vigour and survival by a positive impact on the nutritional[4] and hydratic status of the plant[5] and on soil aggregation[6,7], increasing the reproductive potential, improving root performance and providing a natural defence against invaders, including pests and pathogens.

The most extensively studied AMF fungi are species of the genera *Glomus*, *Gigaspora* and *Scutellospora*. They are obligate symbionts and cannot be cultured axenically without their host system. This is the greatest bottleneck for the progress towards the understanding of the molecular communication between the symbiotic partners. There are many possible reasons why AMF fail to make extensive and continuous growth on a synthetic medium (in the absence of a compatible living host root system). They may have a simple nutritional requirement which, due to lack of our knowledge, has not yet been fulfilled or, it may be necessary to supply some nutrients continuously at low concentration, or else, these fungi may have lost a considerable part of their genomic material and this is acquired upon their interaction with a compatible host (photo-mycobiont metabolism) and/or some part of the genome is in repressed condition and the host supplies the inducer to allow nucleic acid to translate[8].

The growth of arbuscular mycorrhizae in pure culture in the absence of living host roots is a matter of global concern. If these fungi could be grown in culture, many more characteristics might be considered and classification would be simplified. Other fundamental studies would

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also be greatly facilitated, in particular, their genetic modification, selection to obtain superior strains and the biotechnological applications. Axenic culture has now become a subject of interest to commercial companies which envision the possibilities of producing high-quality inoculum under controlled conditions.

Researchers from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have isolated a novel endophytic fungus (Figure 1) which mimics capabilities of typical AMF studied so far. This fungus promises to serve as a substitute of AMF to overcome a long-standing enigma of science.

Piriformospora indica: A new root symbiont

The axenically cultivable plant growth-promoting root endophyte, *Piriformospora indica*, has been characterized in collaboration with several European scientists[9]. Based on the 18S rDNA analysis and the ultra structure of the septal pore, its phylogenetic relationship is within the Hymenomycetes (Basidiomycota, Figure 2).

P. indica tremendously improves the growth and overall biomass production of different plants, like herbaceous mono- and dicots, trees, including medicinal plants (*Bacopa monniera*, *Artemesia annua*), and several economically important crops (Figure 3)[10,11]. Pronounced growth promotional effects were also seen with terrestrial orchids[12] (Figure 4). The fungus promises to be a potential source for colonizing the orchids, their better growth and higher rate of survival of seeds [13]. A recent 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 (pers. commun. G. Kost and co-workers, Marburg, Germany).

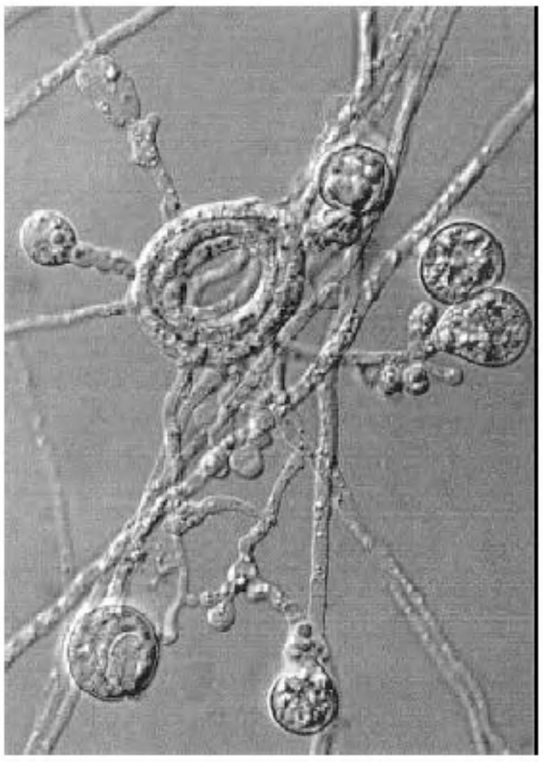

Figure 1. A typical fungus growth on solidified Kaefer[50] medium. Mycelial mat shows the highly coiled hyphae and young and mature chlamydospores. Fungus was incubated at 28 ± 2°C for 14 days.

Physiological and functional identity

P. indica enters 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. Like AMF, 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). Interestingly, the host spectrum of *P. indica* is very much alike AMF. *P. indica* colonizes the roots of host plants as diverse as *Zea mays* L., *Nicotiana tabacum* L., *Petroselinum crispum* L., *Populus tremula* L., *Setaria italica* L., *Oryza sativa* L., *Sorghum vulgare* L., *Triticum sativum* L., *Glycine max* L. Merr., *Cicer arietinum* L., *Solanum melongena* L., *Artemissia annua* L. and *Bacopa monniera* L. Wett.[10,11]. Also like AMF, *P. indica* does not colonize the members of Brassicaceae and the myc$^-$ mutants of *Glycine max* and *Pisum sativum*[11]. This phenomenon may be connected with some identical functional aspects as indicated by the serological data (ELISA, Western blotting, immunofluorescence and immunogold labelling). Data showed close similarities between AMF and *P. indica*[14–16] (Tables 1–3). One striking difference is that unlike AMF, the host range of *P. indica* also includes terrestrial orchids *Dactylorhiza purpurella* (Steph's.) Soo, *D. incarnata* L. Soo, *D. majalis* (Rchb. f.) Hunt & Summerh and *D. fuchsii* (Druce) Soo[11–13].

Western blot analysis showed that the antiserum raised against *P. indica* could strongly recognize four epitopes of *Glomus mosseae* soluble protein antigens. These were of the same molecular weight as in *P. indica*. The high molecular weight of these protein bands raises the possibility that the serological homologies between these two fungi may be associated with the glycosylated components of the proteins. The identification of such serologically specific fractions (proteins, glycoproteins) is important for the future production of specifically targetted antibodies. *G. mosseae* antiserum showed very low cross-reactions with *Scutellospora gilmorei* and *Gigaspora gigantea*. This indicates that the antiserum could differentiate between the Zygomycetous fungi at generic level, however, it was not very specific at species level as it recognized *G. intraradices* significantly. This may be due to the presence of related epitopes at species level, which cannot be differentiated due to the polyclonal nature of the antiserum. *G. mosseae* antiserum also showed almost

similar amounts of cross-reactions with *Gi. gigantea* and *Sc. gilmorei*, confirming that the morphological similarity is paralleled by antigenic similarity. The antiserum strongly recognized *P. indica* antigens, which indicated a serological relationship between these two fungi[17].

Biological hardening agent

A disadvantage of micropropagation, the most powerful technique for cloning plant species, is the high mortality rate during the transfer of *in vitro* plantlets to *ex vitro*[18]. Inoculation of plantlets with AMF during acclimatization has been recommended for reducing the stress of acclimatization, providing faster growth and better establishment of micropropagated plants[19]. *P. indica* promises to be an excellent candidate for biological hardening of micropropagated plantlets as the fungus rendered more than 90 per cent survival rate of the transferred plantlets of (*Nicotiana tabacum* L. and *Bacopa monniera* L. Wett)[20,21]. Recent experiments on the tissue culture-raised coffee (*Coffea arabica* L.) plants have shown very promising results (experiments conducted at Mysore, by Sreenath, data

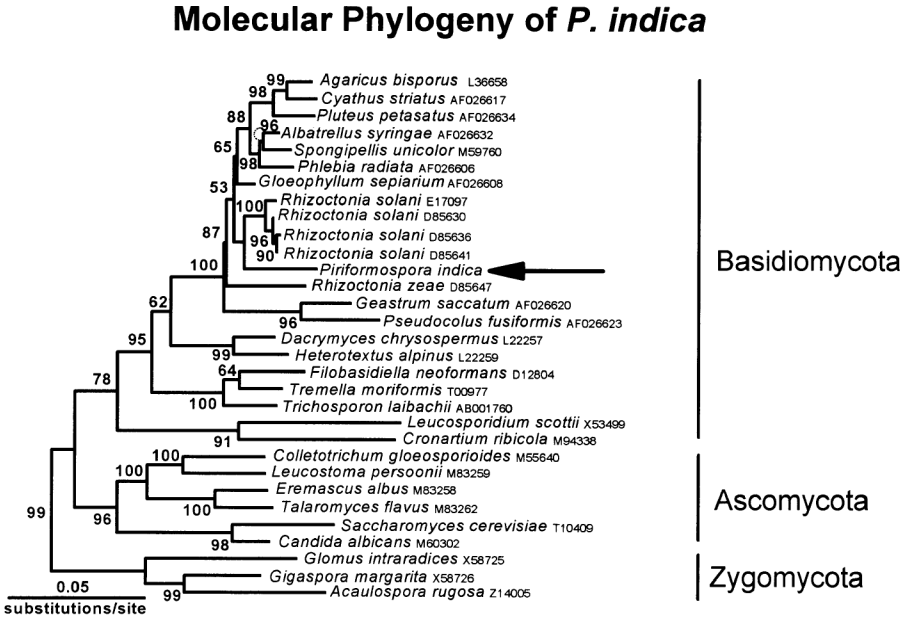

Figure 2. Maximum-liklihood tree estimated by the quartet puzzling method as implemented by PAUP 4.02b2a on 18S rDNA sequences showing phylogenetic relationships between *Piriformospora indica* and other representatives of the Basidiomycota. Branches with support values between 55% were collapsed. Puzzling support indices are shown at each branch[11].

Figure 3. Maize plants (*Zea mays* L.) were grown in surface-sterilized plastic pots containing washed expanded clay. Pots on the left were supplied with dead fungal biomass and those on the right were treated with freshly grown fungus. The rate of inoculum was 1 per cent (w/v). Photographs were taken after 4 weeks.

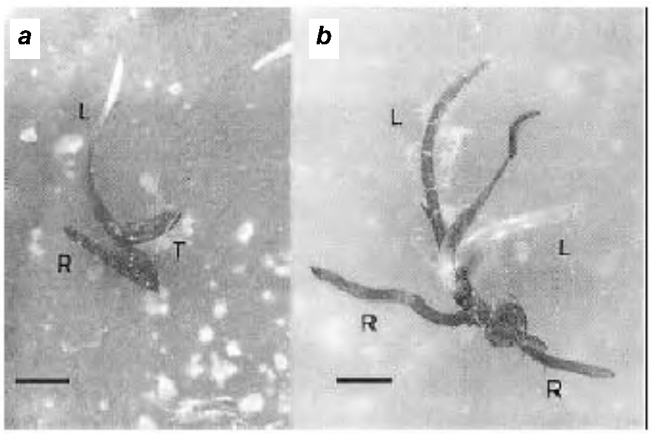

Figure 4 ***a, b.*** *Dactylorhiza maculata* was asymbiotically pre-grown for 2 years and then inoculated with *Piriformospora indica*. ***a***, Control plants without fungus, differentiating only short roots (R), leaf (L) and tuber (T); ***b***, symbiotic plant interacting with the fungus for 3 months with well-developed roots (R), leaves (L) and no tuber (T).

unpublished). Thus, plant industries dealing with tissue culture-raised plantlets can look forward to application of *P. indica* to overcome the 'transient transplant shock' and better development of the plants on transfer to the field.

Phosphate mobilization and transport

AMF colonize the root cortex to obtain carbon from their host plant, while assisting the plant with the improved and rapid uptake of phosphorus and other mineral nutrients of low mobility from the soil, and their translocation to the host root[22]. This is beneficial to the plant as phosphorus is essential for plant growth and development. Studies on *P. indica* have shown fungal-mediated uptake of radiolabelled phosphorus from the medium and its translocation to the host in an energy-dependent process, evident by a sharp increase in its content in the shoot[11]. *P. indica* produces significant amounts of acid phosphatases for the mobilization of a broad range of insoluble, condensed or complex forms of phosphate, enabling the host plant the accessibility of adequate phosphorus from immobilized reserves in the soil (Thomas and Varma, unpublished data). The expression of a phosphate repressible isoform of phosphatase, whose expression was dependent on the external phosphate supply, gave a direct evidence for the involvement of this enzyme in the phosphate metabolism[17,23]. The molecular mechanisms underlying the uptake and translocation of nutrients, especially phosphate, are to be investigated. Because of the absence of axenic cultures in Glomales, such studies are difficult with AMF.

Development of transgenics with *P. indica*

In the absence of axenic culture of AMF, not much progress has been made to genetically engineer them for commercialization in agriculture, horticulture and forestry. Since AMF interact with roots of a wide variety of plants, foreign gene expression in these fungi could exert a profound effect on the root system. *P. indica* mimics most of the beneficial characteristics of AMF studied so far[10,11], and is easily culturable on complex or synthetic media[9]. Thus, it displays immense potential to be utilized as a biological tool to provide the plant factors required for its growth, protection from pests, and for relieving stress conditions such as those due to acidity and heavy metal toxicity. *P. indica* is the most suitable candidate, based upon its wide host range, ease with which it colonizes and its hardiness in surviving adverse environmental conditions, to express foreign genes of importance to the host plants in a symbiosis-specific manner and confirm its benefit for biocontrol.

The protoplasts of *P. indica* have been successfully isolated. Molecular studies on *P. indica* have shown constitutive and high expression of *Pitef1*, encoding the translation elongation factor EF-1α throughout the fungal development (Philipp Franken and co-workers, MPI, Germany, per. commun.). Thus, the *Pitef1* promotor could be a good tool to construct vectors for the development of a transformation system for *P. indica*. This opens an important possibility to improve symbiosis by transgenic manipulation of the fungal component through the introduction of desirable genes. It would be interesting to see the effects on the interaction caused by the introduction of genes, for example, coding for cell wall degrading enzymes. The probes already isolated from an organism producing high amounts of cell wall degrading enzymes could prove very useful and help us to understand to what extent the production of hydrolytic enzymes play role in the plant–fungus compatibility[24].

In recent years, green fluorescent protein (GFP) has been developed as a reporter for gene expression, a marker for sub-cellular protein localization, a tracer of cell lineages and a label to follow the development of pathogens within the host plants[25,26]. As a marker system,

Table 1. Cross-reactivities of the polyclonal antiserum raised against *Piriformospora indica* as determined by ELISA, the method described by Hahn *et al.*[51]. Absorbance is given as mean of three replicates ± standard deviation

Mycobionts	OD_{405} pAb (dilution 1 : 1600)
Piriformospora indica	0.595 ± 0.03
Glomales	
Glomus intraradices	0.429 ± 0.005
Gigaspora margarita	0.408 ± 0.005
Gi. gigantea	0.460 ± 0.002
Scutellospora sp.	0.398 ± 0.002

Table 2. Summary of the performance of antiserum raised from macerated spores of *G. mosseae* observed against *P. indica*, *Sc. gilmorei* and *Gi. gigantea*. Western blot (WB) technique was followed as described by Towbin *et al.*[52]

Antigen	ELISA	WB
Glomus mosseae	+	+
Scutellospora gilmorei	–	–
Gigaspora gigantea	–	–
Piriformospora indica	+	+

Table 3. Summary of the performance of antiserum raised from macerated spores and hyphae of *P. indica* and cross-checked against *G. mosseae*, *Sc. gilmorei* and *Gi. gigantea*

Antigen	ELISA	WB
Piriformospora indica	+	+
Scutellospora gilmorei	+	–
Gigaspora gigantea	+	–
Glomus mosseae	+	+

GFP, which accepts excitation energy from luciferases or photoproteins[27], has several advantages[28,29] over other existing reporters[30]. Thus, it would be now possible to transform *P. indica* protoplasts and to apply such transformed strains to rhizospheres to investigate colonization patterns, population dynamics or dispersal under natural conditions. The micro-environmental relationships[31] and the demographic factors[32] could also be studied.

Other theoretically important experiments for transgenic manipulation of *P. indica* may involve gene(s) for destroying pesticide residues in soil[33], for nitrate reduction[34], or for heavy metal resistance[35]. In such designs of genetically modifying the fungus, it will be important to avoid non-target effects that could disrupt the rhizosphere ecology.

The obvious extension of transformation experiments involves the identification of genes and regulatory sequences which might positively influence symbiosis between the fungus and the host. Fungal genes important to root development and/or plant survival under biotic and/or abiotic stress conditions warrant urgent identification.

Immobilization of the endosymbiont

Immobilized spores/hyphae serve as a potential source of viable inoculum. The immobilization procedure can preserve the physiological properties and promote regeneration of mycelium and formation of mycorrhizas[36]. It is well established to produce masses of ectomycorrhizal fungi in a fermenter and entrap the mycelium in alginate beads[37,38]. There are only a few reports on the immobilization of AMF. Moreover, the methods described are either time consuming[39,40], or the immobilized propagules were unstable with time and could not be stored for long[41].

In our recent experiments in which spores and hyphae of *P. indica* were immobilized in alginate beads, the former were found to retain the germinating capacity even after 90 days storage at low (4°C) and room temperature (30°C), when used as an inoculum in pot culture experiments interacting with *Sorghum vulgare* as host[42].

These findings ascertain that the entrapment of fungal propagules inside alginate beads stabilizes the infective capacity of the fungus and promises its use as a viable inoculum for various biotechnological applications and feasible long-distance transportation. Efforts to make alginate beads for dual culture loaded with *P. indica* and terrestrial orchid seeds are in progress. It is well documented that in their initial developmental steps, orchids are totally dependent on mycorrhizal interaction, because there is no chlorophyll in the germinating seeds. The combination shall make a breakthrough for the commercial propagation of terrestrial orchids, as normally the survival rate of orchid seeds alone is extremely poor[13].

Mass scale production: The fermentation technology

Producing large quantities of pure inoculum of the mycorrhizal fungi, free from pathogens, with high infectivity potential, easy to be transported across the country, and assessing their use in field and green-house conditions are substantial in view of the wide range of benefits which they accrue. AMF propagules are difficult to produce on a commercial scale. Presently, these fungi can be grown with host plants in pot culture containing soil[43], sand[44] or expanded clay[45]. They have also been grown by using hydroponics[46], aeroponics[47] and root organ culture[48], all of which are not a cost-effective proposition[49,50].

P. indica can be propagated on several economically viable synthetic media, viz. Kaefer and potato dextrose media. Fermentation technique should be optimized to device a simple, cheap and commercially viable technique for the mass scale inoculum production for application in agro-forestry and horticulture. Especially for the better establishment of tissue culture-raised plants, fungal inoculum will be much needed in the plant industry.

Summary

Abiotic factors, viz. climate, availability of nutrients, and water are normally considered to have the greatest influence on the plant productivity. But the biotic factors also have eminent impact on the productivity of plants. This is obvious concerning the plant pathogens. However, other micro-organisms, which are not that obvious, also determine the productivity, especially those interacting with the roots. They are essential partners for most of the terrestrial plants in agriculture, horticulture and forestry, and play a vital role in their fertility and survival. In this scenario, mycorrhizal fungi, especially AMF, play an important and integral part in plant health. The growth promotional effects of *P. indica* on a compatible photosymbiont do not exclusively demand the physical contact of the mycelium, but could also be realized with the treatment of the host with small quantities of the culture filtrate. The culture filtrate was also effective in inhibiting the growth of a few potent root fungal pathogens tested. The exact biochemical nature of the stimulatory and inhibitory factor(s) is yet to be deciphered.

The application of modern phylogenetic and immunological techniques has provided new insights to understand further aspects of mycorrhizal functioning. Studies indicated that these techniques offer a better choice for the identification of the AMF than the conventional methods being routinely used. Recent experiments have opened up several directions which can be explored to fill-up the lacunae in understanding the molecular aspects of arbuscular mycorrhizal research. The cross-reaction of *P. indica* antisera with epitopes of *G. mosseae*, high-

lighted the same molecular weights of the corresponding bands in the Western blot. These findings along with the almost similar host range of the two fungi, gave a strong indication towards their identical functional aspects. Antibodies against such purified epitopes/peptides can generate more specific response and information in a short time span. These antibodies could be employed more efficiently to study the symbiotic fungi in the colonized plant roots and could be used as markers to study the molecular basis of symbiosis in *P. indica*. If any sequencial/structural/chemical homology is observed between these peptides of *P. indica* and *G. mosseae*, the results may be correlated with the AMF using *P. indica* as a model fungus.

The future genetic research with *P. indica* would focus on clarifying mycorrhizal symbiosis through genetic manipulation of the fungal component by introducing genes of desirable attributes, such as those coding for fluorescent markers (GFP), for tracing their distribution and dispersal in nature, as well as for studying their colonization patterns and demographic factors during the symbiotic status. It would be necessary to obtain fundamental information on relevant genes and their expression by establishing and screening the cDNA and genomic libraries. Molecular genetic mechanism(s) underlying nutrient uptake, especially phosphate, and its transmembrane translocation are to be investigated. Likewise, the photosynthate translocation and carbon assimilation mechanisms are also to be elucidated. It would be relevant to identify and characterize the monosaccharide-uptake system in *P. indica* by the isolation of cDNA(s) encoding the monosaccharide transporter(s) and expression pattern of the corresponding gene(s) in *P. indica* grown under asymbiotic condition. These studies hopefully lead to a better understanding of the molecular basis for plant–microbe interactions and evolution of plant–fungus associations.

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