

The growth of tomato seedlings inoculated with co-cultivated *Piriformospora indica* and *Bacillus pumilus*

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Abstract A novel co-culture method using the beneficial root endophytic fungus *Piriformospora indica* and a plant-growth promoting rhizobacterial strain of *Bacillus pumilus* to promote the growth of tomato seedlings in plug trays, is described. Coconut water, a waste product from the coconut industry, was used as medium for co-cultivation of the two biological agents. During the co-culture with the fungus, the bacterial strain showed a similar growth rate to that in monoculture in fresh autoclaved coconut water. In contrast, co-culture in potato dextrose broth (PDB), a medium routinely used for cultivation of the endophytic fungus, did not support bacterial growth. Inoculation with the co-culture or a mixture of *P. indica* and *B. pumilus* promoted tomato seedling growth significantly when compared with individual application of the two biological agents. No difference was observed with respect to the degree of root colonization in tomato seedlings whether it was done with a monoculture or a co-culture with rhizobacterium. The co-culture system developed reduces the cost of inoculum production as it uses coconut water, a cheap and locally available waste product and a single fermentation vessel. Further research is required to know the endophytic nature of the rhizobacterial strain and its possible role in helping the fungus in root colonization.

Keywords Co-culture · *Piriformospora indica* · PGPR · *Bacillus pumilus* · Coconut water · Plant-growth promotion · Tomato

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1 Introduction

The production of good quality seedlings with high seedling vigour is one of the important factors in production systems for vegetables. The use of healthy seedlings with well established root systems helps to avoid transplantation shock when the seedlings are transferred to the field from the nursery. Raising seedling using plug trays (pro-trays) allows the grower to establish seedlings with optimal spacing and uniform physiological status prior to transplanting (Vavrina 1998). Plug tray transplants are commercially used for crops like chilli, tomato, brinjal, cauliflower, cabbage, broccoli, celery, cucumber etc. The use of biological agents at the nursery production stage is advantageous as root colonization by beneficial microbes, both fungi and bacteria results in the transfer of “bio-primed” seedlings to the field. Plant growth promoting rhizobacteria usually colonize the root system, both externally as well as endophytically. They positively affect plant health, leading to increased growth and improved yields (Klopper et al. 1980; Hass and Defago 2005). Several of these growth promoters possess additional capabilities such as the biological suppression of plant diseases and insect pests (Lugtenberg and Kamilova 2009). Biological amendment with PGPR inoculants for the production of vegetable seedling have been reported by several workers (Gagne et al. 1993; Nemeč et al. 1996; Kokalis-Burelle et al. 2002; Russo 2006a, b; Russo and Perkins-Veazie 2010). The survival and root colonization pattern in tomato of several rhizobacteria in soil-less growth medium was studied by Yan et al. (2003), while the value of the fungus *Trichoderma harzianum* at the nursery stage has been studied by Chowdappa et al. (2013).

Piriformospora indica, a fungus belonging to the Sebaciales in the Basidiomycota is a root colonizing endophyte with a wide host range and plant growth-promoting ability. The fungus can be cultivated on complex or minimal media (Verma et al. 1998; Weiss et al. 2004). This fungus may

also confer resistance against several biotic and abiotic stresses including root and leaf fungal pathogens, as well as promoting early flowering or enhanced seed production (Oelmüller et al. 2009; Franken 2012; Varma et al. 2012). Root colonization by the fungus induced improved secondary metabolite production in the medicinal plant *Centella asiatica* (Satheesan et al. 2012) and also increased gel content, phenol content, aloin content and radical scavenging capacity in *Aloe vera* (Sharma et al. 2014). Finally, *P. indica* has been reported to act as a growth promoter for tomato (Fakhro et al. 2010).

The efficiency of bio-inoculants can be increased by using more than one biological agent that can work together thereby increasing the action spectrum (Janisiewicz 1988; Pierson and Weller 1994; Vidhyasekarn and Muthamilan 1995; Schisler et al. 1997; Slininger et al. 2010; Schisler et al. 2011). *P. indica* has been applied with beneficial bacterial strains such as PGPR as a mixed inoculum for mung bean and tomato. This resulted in improved growth response and disease suppression ability (Sarma et al. 2011; Kumar et al. 2012). Improved plant growth in chick pea has also been reported for the combined inoculation involving *P. indica* and the phosphate solubilizing bacterium *Pseudomonas striata* (Meena et al. 2010). Positive effects of dual inoculation of *P. indica* and the mycoparasitic fungus *Trichoderma harzianum* have been found on the growth of black pepper in tissue culture (Anith et al. 2011).

Co-culture systems containing a combination of a plant-growth promoting/plant-defense enhancing fungus and a bacterium have not been reported to date. In the present study, it was decided to employ a plant-derived organic substrate for the cultivation of the microbial agents to minimize costs. Coconut water in the intact coconut is free from microbial contamination and is highly nutritious, rich in many amino acids, vitamins and minerals (Nandakumar 1990). Coconut water from the mature coconut is a waste product from processing the nuts, though it has been used for growing microbes and in plant tissue culture (Anandaraj and Sarma 1997; Gopal et al. 2006; Survase et al. 2007; Unagul et al. 2007; Prabhakaran et al. 2008). It is an excellent multiplication medium for plant growth promoting rhizobacteria (Anith 2009). In the present paper we describe an efficient co-cultivation protocol involving the fungus *P. indica* and the plant-growth promoting rhizobacterial strain of *Bacillus pumilus* in autoclaved coconut water and the effect of the co-cultured inoculum on the growth of tomato seedlings.

2 Materials and methods

2.1 Cultivation of fungal and bacterial strains

The endophytic fungus *Piriformospora indica*, obtained from Dr. Ajit Varma, Professor emeritus at Jawaharlal Nehru University, New Delhi, India was routinely cultivated on Potato

Dextrose Broth (PDB; pH 6.5) or Potato Dextrose Agar (PDA; pH 6.5) under conditions described earlier (Anith et al. 2011). During the course of the present study, cultivation was also performed on coconut water agar (CWA) and autoclaved coconut water (ACW) providing similar incubation temperature as in the case of PDA and PDB. For this, fresh coconut water was procured from a local coconut processing facility and filtered through muslin cloth to get rid of the suspended particles and debris. 100 ml of coconut water were transferred to a 500 ml Erlenmeyer flask, the pH was adjusted to 6.5 and the solution was sterilized by autoclaving at 121 °C for 20 min. For preparing CWA, 2 % agar were added before autoclaving.

Bacterial cultures used in the study included PGPR strains of *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* available at the Department of Agricultural Microbiology, College of Agriculture, Vellayani (India). *Bacillus* strains were grown on Nutrient Agar/broth (Anith 2009) and the *Pseudomonas* strain on King's medium B agar/broth (Anith et al. 2002) at 28 °C. Broth cultures were incubated in a shaker at 150 rpm for 48 h.

2.2 Growth of *P. indica* on coconut water based media

For finding out the suitability of coconut water based media for the growth of *P. indica*, the fungus was cultivated on CWA and in ACW. For measuring the growth of *P. indica* on CWA, a mycelial disc (8 mm dia) of *P. indica* from freshly grown PDA plate was cut out with a sterile cork borer, placed in the center of a CWA plate and incubated at 28 °C. The diameter of the radial growth of the fungus was measured at regular intervals. In parallel, the growth of the fungus was analyzed on PDA plates. Ten replicates were analyzed for each medium.

Fungal growth in liquid medium (ACW) was measured by quantifying the mycelial wet and dry weight after an incubation period of 15 days under shaking at 90 rpm. To 100 ml of ACW a mycelial disc (8 mm dia) of *P. indica* was aseptically inoculated and incubated with agitation (90 rpm). After 15 days of growth, the mycelium was collected by filtration through a filter paper (Sartorius No. 292) and the fresh weight of the mycelium was determined. The collected mycelium was dried in an oven at 55 °C till it achieved constant weight and the dry weight was determined. The results were compared with fungal growth in PDB under the same conditions. Five replicates were examined per type of medium.

2.3 Testing for in vitro antagonism between *P. indica* and bacterial strains

Dual culture plate assays were conducted on PDA and CWA as described elsewhere to assess whether the bacteria displayed antagonism against *P. indica* (Nair and Anith 2009). A mycelial disc (8 mm dia) from a freshly grown

P. indica culture on PDA was placed in the centre of a Petri dish with agar medium and incubated at 28 °C for 5 days. Single colonies of the *Bacillus* strains and of *Pseudomonas fluorescens* were grown on Nutrient Agar and King's medium B, respectively. After 5 days of incubation of the *P. indica* plates, a heavy inoculum from a single colony of the bacterial strain was applied with an inoculation loop as a band of 1.5 cm length equidistantly on two opposite edges of the agar plate with the fungus, so that two independent measurement on fungal growth inhibition could be taken from a single plate. Four plates were examined for each of the bacterial strains. The plates were incubated at 28 °C and observations on the inhibition of fungal growth by the bacterial strains were made by measuring the zone of inhibition 5 days after the inoculation with the bacteria. Plates containing the fungus alone served as the control.

2.4 Co-culture experiment

Since *Bacillus pumilus* showed no antagonism against *P. indica* on both CWA and PDA medium, it was selected for the co-cultivation experiment. *P. indica* was initially grown in PDB or ACW for 15 days as described above. *B. pumilus* was streaked out for single colonies on a nutrient agar plate. Cells from a single colony were pooled in one ml of sterile distilled water (population density of 2×10^6 cfu/ml) and 200 μ l of the bacterial suspension was aseptically added to flasks of PDB and ACW wherein *P. indica* had been growing since 15 days. The initial number of the bacteria added to the flasks was determined by dilution plating on nutrient agar medium immediately after inoculation. The flasks were further incubated under agitation (150 rpm) for 72 h and the population of the bacteria was determined at 24 h intervals by dilution plating on nutrient agar medium. The bacterial population from five flasks was independently assessed for both growth media. Growth of the bacteria in fresh PDB and ACW was taken as baseline to determine the efficiency of the co-culture in supporting bacterial growth.

2.5 Plant growth experiment

Seeds of the tomato variety Vijay were surface sterilized in 1 % sodium hypochlorite solution for 3 min. and washed thrice with sterile distilled water under aseptic conditions. Exfoliated vermiculite (particle size 0.5 to 1 mm; pH 6.5) was used as planting medium for the growth promotion experiment. It was sterilized by autoclaving at 121 °C for 1 h each for three consecutive days. Plastic plug trays with cavities of 5 cm diameter and 5 cm depth were used for maintaining the plants. Plant growth promotion by the fungus grown in PDB or ACW, respectively, by co-cultured fungal-bacterial suspension in ACW and by the bacterial strain grown in ACW was compared. Dual inoculation of the fungus and the bacterial

strain grown separately in ACW was also included for comparison. The control treatment involved no inoculation. The experiment was designed in CRD with three replicates of 10 seedlings each.

Fungal mycelium was incorporated into the planting medium before filling it in the plug trays as described elsewhere (Anith et al. 2011). For this the mycelium of *P. indica* grown in PDB and ACW in 100 ml medium in 500 ml flasks for 15 days was collected by filtering the suspension through muslin cloth. It was weighed and mixed thoroughly with sterile vermiculite so as to get a 1 % (w/v) final concentration of the fungal mycelium in the vermiculite.

For inoculation with bacteria alone, *B. pumilus* was used after growth in ACW for 48 h at 28 °C with agitation to a density of 8×10^8 cfu/ml. No incorporation of the bacterial culture into the planting medium was done prior to planting. However, one ml of the bacterial culture was added to the planting hole in the vermiculite after depositing the seeds.

Co-culturing of the fungus and the bacteria was done as described earlier. Forty-eight hours after the addition of the bacteria to the flask containing the fungus grown in ACW, the mixture was filtered through muslin cloth and the fungal mycelium was collected and its fresh weight was determined. The incorporation of the fungal-bacterial mixture in vermiculite (1 % w/v) was performed as described above. No further supplementation of bacterial culture was done, as the co-cultured fungal mycelium contained bacterial cells at a population level of 9.23×10^8 cfu/g (on a wet weight basis).

Mixed inoculation of the fungus and bacteria was performed as follows. Mycelium of the fungus grown in ACW for 15 days was mixed with vermiculite at the rate of 1 % (w/v) as described above and filled in plug trays. *B. pumilus* was grown in ACW for 48 h with agitation in a rotary shaker. After planting the seeds, one ml of the bacterial culture with a density of 8×10^8 cfu/ml was added to the planting hole.

Two seeds were planted in a single cavity of the plug tray. After germination, a single seedling was maintained per cavity. Plants were cultivated in a glass house with 16 h light at 28 ± 2 °C. Seedlings were irrigated twice daily with sterile distilled water. Hoagland's Nutrient Solution (Douds and Schenck 1990) was provided to the seedlings at a rate of 10 ml per plug tray cavity, once in 10 days, starting 1 week after seeding. Plants were kept for 21 days and quantification of plant height, number of leaves per plant and fresh and dry shoot and root weight of the plants was carried out after uprooting the plants.

2.6 Colonization assay

Separate sets of five plants each per treatment described above were kept for colonization studies under greenhouse conditions. After 21 days of growth, five plants from each treatment were analyzed for root colonization by the endophytic fungus

P. indica following procedures described earlier (Anith et al. 2011). The plants were uprooted, washed in running tap water to get rid of the planting medium and the root systems were cut off. The collected roots were cut into pieces of one cm and boiled in 10 % KOH for 5 min., then washed in sterile water followed by neutralization with 2 % HCl. Roots were then stained with 0.5 % trypan blue in lactophenol for a period of 10 min. They were then destained with lactophenol solution for 15 min to remove excess stain. The stained root bits were viewed under a compound bright field microscope and the presence of chlamydospores in the cortex cells documented for each root segment. The percentage of colonization was assessed using the following formula.

% root colonization

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

2.7 Statistical analysis

Statistical analysis for all the parameters was performed using one way analysis of variance (ANOVA) and the means were compared by Duncan's Multiple Range Test, using the statistical package SAS version 8.1 (SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Growth of *Piriformospora indica* on coconut water based media

The average diameter of the fungal mycelium 10 days after inoculation was 6.2 and 5.38 cm on PDA and CWA, respectively (Table 1). The entire surface of the medium in the 10 cm diameter plate was fully covered by fungal mycelium on the

14th or 16th day, respectively, for PDA and CWA. There was slight difference in the colouration of the fungal mycelium on the two media as on PDA the mycelial mat appeared slightly yellowish while on CWA it was almost white. The mycelial fresh and dry weight determined after 15 days of growth in PDB and ACW, respectively, is shown in Table 1. More mycelium was produced in PDB than in ACW.

3.2 In vitro antagonism between *P. indica* and bacterial strains

Dual cultivation of the fungus and bacterial strains showed that *B. pumilus* had no antagonistic effect on the fungus both on PDA and CWA (Table 2). The maximum zone of inhibition of fungal growth was observed with *Bacillus amyloliquefaciens* both on PDA and on CWA.

3.3 Co-culture experiment

Since *B. pumilus* had no antagonistic effect on the fungus, this strain was taken for co-cultivation. When 15 day-old cultures of the fungus in ACW and PDB, respectively, were inoculated with the bacteria, it became obvious that the former medium supported the growth of the bacteria similarly to fungus-free ACW (Table 3). Both monoculture and co-culture in ACW resulted in achieving a population of 10^8 cfu/ml from an initial inoculum of 10^5 cfu/ml. On the other hand, co-cultivation in PDB led to a decline in bacterial population, in spite of the fact that *B. pumilus* did not show significant differences in growth in fungus-free PDB or ACW. The initial pH of the ACW before the growth of *P. indica* was 6.19, while after 15 days of growth of the fungus it has come down to 5.97. Though there was a reduction in pH value, the bacterial strain was able to multiply in it. The final pH after 72 h of growth of the bacterial strain in the co-culture system was 5.95. Since there was only a slight reduction in pH value even after bacterial growth, the fungal mycelium was not adversely affected.

Table 1 Growth of *P. indica* in coconut water based media

Medium	Diameter of mycelial growth (cm)*	Mycelial fresh weight (g)**	Mycelial dry weight (g)**
Agar medium			
CWA	5.38±0.147	–	–
PDA	6.20±0.201	–	–
Broth			
ACW	–	3.59±0.401	0.342±0.069
PDB	–	4.82±0.193	0.416±0.039

* Observations taken after 10 days of inoculation. Mean ± SD of observations from ten replications

** Observations taken after 15 days of inoculation. Mean ± SD of observations from five replications

Table 2 Mycelial growth inhibition of *P. indica* by bacterial strains

Bacterial strain	Zone of inhibition (cm)*	
	PDA	CWA
<i>Bacillus pumilus</i>	Nil	Nil
<i>Bacillus amyloliquefaciens</i>	0.75	0.50
<i>Bacillus subtilis</i>	0.85	0.90
<i>Pseudomonas fluorescens</i>	0.65	0.45

*Mean of eight observations from four dual culture plates. Each plate represents single replication ($n=4$)

Table 3 Population build-up of *Bacillus pumilus* in different media and cultural conditions

Type of inoculation	Population of <i>B. pumilus</i> (cfu/ml)*			
	Time of population assessment			
	0 h	24 h	48 h	72 h
<i>B. pumilus</i> alone				
PDB	4.72×10^5	4.51×10^7	2.65×10^8	2.67×10^8
ACW	2.12×10^5	7.27×10^8	8.50×10^8	5.02×10^8
<i>P. indica</i> and <i>B. pumilus</i> co-culture				
PDB	4.48×10^5	5.5×10^4	4.03×10^4	2.95×10^4
ACW	4.62×10^5	1.15×10^7	2.33×10^8	1.41×10^8

*Mean population from five flasks. Each flask represents single replication ($n=5$)

3.4 Plant growth experiment

Results of the plant growth promotion experiment showed that there were significant differences in plant growth parameters between the effects of the combined application of *P. indica* and *B. pumilus*, both as co-culture and as mixed inoculum, and the application of either *P. indica* or *B. pumilus* alone (Table 4). Inoculation with *P. indica* alone as well as *B. pumilus* alone also improved the plant growth compared to the uninoculated control. Among all plant growth parameters analyzed, the maximum values were obtained in plants treated with either the co-cultured suspension of *P. indica* and *B. pumilus*, or with a mixed inoculum of the two biological agents. With respect to plant height there was no significant difference among the treatments except that the uninoculated control plants were shorter. Treatments with biological agents, when applied singly or in combination, had a positive impact on the growth of the plants when compared with the uninoculated control.

3.5 Colonization assay

Microscopic examination of the root pieces after staining revealed that in all the treatments that included the endophytic fungus, colonization was evident by the presence of chlamydo spores of the fungus in the cortical region of the plant roots. Maximum percentage colonization (37.4 %) was observed with plants treated with the co-cultured inoculum (Table 5). However, there was no statistically significant difference with respect to the percentage root colonization among the different treatments ($p=0.833$). When the root colonization by the endophytic fungus was assessed by examining chlamydo spores in the root cortex cells of tomato, it was found that colonization percentage was similar following application of single, mixed and co-cultured inocula. However, there was a difference with respect to the pattern of chlamydo spore formation in the cortical cells (Fig. 1). Whenever a single inoculation using *P. indica* was performed, the root cortex cells occupied with chlamydo spores were completely filled with many numbers of large sized spores of the fungus. Only very few adjoining cells were filled with spores. However, when mixed or co-cultured inoculations, were used, it was observed that almost all the cells in the cortex region in the colonized roots were occupied by chlamydo spores, but with a different distribution pattern. Most of the cells were occupied by single or comparatively small spores. More research is needed as it is uncertain whether the rhizobacterial strain used also acts as an endophyte and help the fungus to colonize cortical cells. Molecular tagging of the bio-agents and confocal microscopic studies would be necessary to further confirm this.

4 Discussion

The application of biological agents to seedlings in the nursery has several advantages as compared with treatment of the seedlings in the field. Dipping seedling in a suspension of the formulated product during transplantation is cumbersome

Table 4 Growth parameters of portray seedlings of tomato taken after 21 days of planting

Treatment	Biometric observations*					
	Plant height (cm)	Leaf number	Fresh shoot weight (g/plant)	Dry shoot weight (mg/plant)	Fresh root weight (g/plant)	Dry root weight (mg/plant)
<i>P. indica</i> in ACW	8.20±0.25a	4.76±0.29a	0.746±0.03bc	57.13±2.75b	0.117±0.010b	7.69±1.21ab
<i>P. indica</i> in PDB	8.58±0.51a	4.26±0.15b	0.724±0.11bc	57.96±10.55ab	0.127±0.015ab	6.65±1.41bc
Co-cultured <i>P. indica</i> and <i>B. pumilus</i>	9.16±0.20a	4.73±0.15a	0.876±0.01a	67.15±2.10a	0.138±0.005a	8.43±0.98a
Mixed inoculation of <i>P. indica</i> and <i>B. pumilus</i>	9.12±0.77a	4.70±0.20a	0.815±0.07ab	61.60±4.88ab	0.128±0.008ab	7.19±0.63abc
<i>B. pumilus</i> in ACW	9.00±0.65a	4.73±0.23a	0.647±0.02c	43.35±1.33c	0.091±0.002c	5.52±0.32c
Control	6.80±0.50b	3.67±0.23c	0.401±0.05d	30.77±3.35d	0.065±0.006d	3.61±0.29d

* Mean ± SD of three replications having 10 plants each. Figures in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test (DMRT). $p < 0.05$

Table 5 Root colonization in tomato by *Piriformospora indica*

Treatment	Root colonization (%)*
<i>P. indica</i> in ACW	35.4a
<i>P. indica</i> in PDB	35.8a
Co-cultured <i>P. indica</i> and <i>B. pumilus</i>	37.4a
Mixed inoculation of <i>P. indica</i> and <i>B. pumilus</i>	36.7a
<i>B. pumilus</i> in ACW	ND
Control	ND

*Observation taken 21 days after planting. Mean of five replications having one plant each ($n=5$). From each plant 100 root segments were assayed for determining the presence of chlamydo spores in the root cortex cells. Figures in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test (DMRT). $p < 0.05$ ND Not detected

and labour intensive. If seedlings are already treated with the biological agents while in plug trays, the further multiplication of the agents may be expected during crop growth without additional application (Yan et al. 2003; Russo 2006a, b; Russo and Perkins-Veazie 2010). The endophytic fungus *P. indica* is usually mass multiplied in potato dextrose broth for plant growth promotion experiments and inoculum preparations

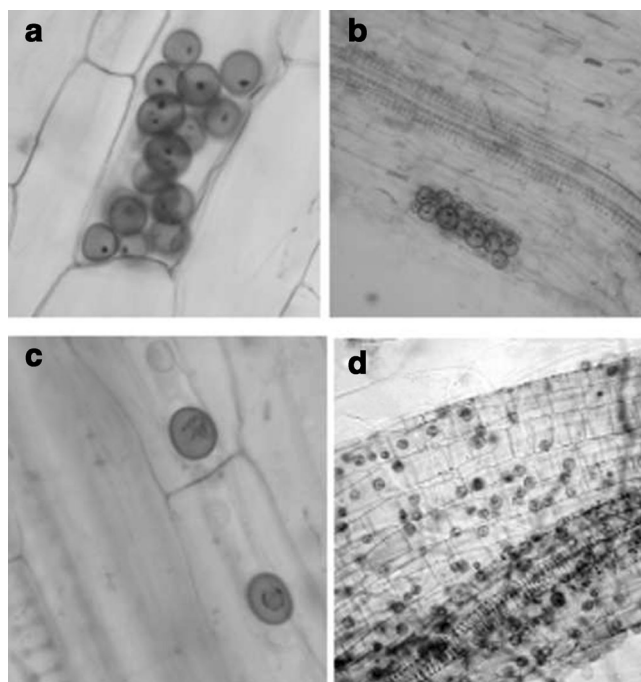


Fig 1 *Piriformospora indica* colonization and chlamydo spore formation pattern in the root cortex of tomato seedlings. *Upper panel*: Root colonization in plants treated with *P. indica* as a single inoculum. (a- Enlarged view of a single cell filled with many numbers of the chlamydo spores; b- Root segment showing chlamydo spores within the cells with very few cells occupied by the spores). *Lower panel*: Root colonization in plants treated with co-culture of *P. indica* and *Bacillus pumilus*. (c- Enlarged view of cells filled with single chlamydo spores; d- Root segment showing chlamydo spores within almost all the cells, occupied singly)

(Fakhro et al. 2010; Anith et al. 2011; Sarma et al. 2011; Kumar et al. 2012). In the present study, the fungus grew well on solid CWA, and autoclaved coconut water (ACW) was found to be suitable for mass multiplication of *P. indica*. The growth of the fungus in ACW was comparable to that in potato dextrose broth, a routine medium for its culture. During the course of evaluation of ACW as a medium for cultivation of the endophytic fungus, the mycelial growth was separated from the liquid portion by straining through a muslin cloth and the liquid portion was left behind in an unsterile vessel. It was noticed on the next day that the clear liquid turned turbid as a result of multiplication of bacterial contaminants. This prompted us to use the left over coconut water that was used for cultivation of *P. indica* for 15 days, as a medium for growth of bacterial biological agents. The liquid portions from several such flasks were collected aseptically after removing the *P. indica* mycelium and evaluated for the growth of PGPR strains in them. All the bacterial strains tested, three *Bacillus* and one *Pseudomonas* strain, were able to multiply in the liquid medium (data not presented). Cultivation of the PGPR strains along with the endophytic fungus in a co-culture system was then contemplated after studying their in vitro interactions.

Co-culturing of biological agents in the same fermentor system has been proposed previously (Slininger et al. 2010) for bacterial antagonists to control diseases in stored potatoes. Dual culture has been used to understand antagonism between different biological agents (Li et al. 2002; Anith et al. 2003; Aravind et al. 2009). The endophytic root symbiont *P. indica* induced various reactions with rhizobacterial strains. Many had a neutral response, but some displayed inhibitory to stimulatory reactions to *P. indica* when co-cultivated on agar plates (Varma et al. 2012). In the present study, the selection of the rhizobacterial strain was done after an in vitro assay for antagonistic interactions against the fungal endophyte on different solid media by the dual culture plate method. Since the PGPR strain *B. pumilus* showed no antagonism against *P. indica*, it was used for co-cultivation. Co-cultivation in ACW supported the growth of the bacterial strain and bacterial growth was comparable to that in a monoculture in ACW, even in the presence of the fungal mycelium. It is inferred that the ACW still contains sufficient amount of nutrients that could support the growth of a bacterial strain even after supplying nutrients for the growth of the fungus for 15 days. The initial pH of the ACW before the growth of *P. indica* was 6.19, while after 15 days of growth of the fungus it has come down to 5.97. Though there was a reduction in pH value, the bacterial strain was able to multiply in it.

In the present study the two biological agents used were compatible to each other as shown by the dual culture assay. Whenever there is antagonism among the different biological agents it is not advisable to use them for mixed inoculation on crop plants. However, incompatibility of the inoculants could

to some extent be circumvented by temporal separation in application to the root zone of the crop plants (Anith et al. 2011). Though there was an antagonistic reaction against *P. indica* by *Trichoderma harzianum*, it was proven that they could be used as dual inoculants in black pepper, if the root endophyte was applied at an early hardening stage of the tissue cultured black pepper, followed by the application of the mycoparasitic fungus during transplantation to the field. Root colonization by *P. indica* in tomato positively influence plant growth as well as improves defensive capacity against fungal and viral pathogens (Fakhro et al. 2010). Strains of *B. pumilus* are reported to have plant growth promotional effects and induction of systemic resistance (ISR) in tomato (Benhamou et al. 1998; Yan et al. 2002). Combining the fungal and rhizobacterial biological agents, therefore, would have additional benefits provided to the tomato plants than when applied singly. Results of the current investigation suggest that mixed inoculation and inoculation with the co-culture of *P. indica* and *B. pumilus* are more efficient than single inoculation of the biological agents for improving seedling growth in tomato. Previous reports involving other crops also supported the idea of co-inoculation of *P. indica* with beneficial bacteria to achieve greater plant growth (Meena et al. 2010; Sarma et al. 2011; Kumar et al. 2012).

To our knowledge, this is for the first time that a co-cultivation system is proposed using the combination of a beneficial fungus and a rhizobacterium. Further investigations are needed to discover if the currently devised co-cultivation system of *P. indica* and *B. pumilus* leads to biofilm formation and if so, how the biofilm affect the rhizosphere. Another worthwhile investigation would be to study the effect of the co-cultivated inoculum on the suppression of plant diseases in crops since the endophytic fungus *P. indica* possesses control potential against many soil borne and foliar pathogens, and the rhizobacterial strain *B. pumilus* antagonizes several soil-borne plant pathogens (Anith et al. 2004; Oelmüller et al. 2009; Fakhro et al. 2010; Franken 2012).

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