Role of growth media on the phytopromotional potential of symbiotic fungus *Piriformospora indica*

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**Abstract**

*Piriformospora indica* biomass generated in different growth media Rose Bengal medium, Kaefer's Medium, Enriched Soil Medium, Malt extract Medium and Czapek Dox was quantified and their bioinoculum potential was compared using *Vigna radiata* (mung bean) as test plant. Plant chlorophyll content in response to inoculations was lowest in Rose Bengal medium (2.772 mg plant\(^{-1}\)) and highest due to inoculum produced on Enriched soil Medium (3.694 mg plant\(^{-1}\)). The highest nitrogen content (19.260 mg plant\(^{-1}\)) was recorded by inoculum produced on Kaefer's Medium followed by Enriched Soil Medium (19.123 mg plant\(^{-1}\)), ME (18.19 mg plant\(^{-1}\)) and CD medium (17.71 mg plant\(^{-1}\)). The highest plant phosphorus uptake was registered in Enriched Soil Medium (17.153 mg plant\(^{-1}\)) followed by Kaefer's Medium (17.023 mg plant\(^{-1}\)). Maximum dry weight of plants was observed by inoculation with fungus cultured in Kaefer's Medium (3.416 g pot\(^{-1}\)) and Enriched Soil Medium (3.349 g pot\(^{-1}\)). Thus, growth medium used for the culture of fungus can influence its bioefficacy as plant growth promoting agent and *Piriformospora indica* can be grown on cost effective and simple mass multiplication medium which could augment its usage for commercial purposes.

**Key words**

Growth media, Moong bean, *Piriformospora indica*, Phytopromotion potential

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**Introduction**

*Piriformospora indica* is a wide-host, root-colonizing endophytic phytopromotional, fungus mimicking the capabilities of arbuscular mycorrhizal fungi (AMF) (Linares *et al.*, 2013). This endosymbiont allows the plants to grow under extreme physical and nutrient stress (Johnson *et al.*, 2013). The fungus belong to order Sebacinales in Basidiomycota and can be cultivated on complex and minimal substrates unlike other known obligate symbiotic AM fungi (Rai *et al.*, 2004). The fungus is of value for basic research, as well as, biotechnological applications because it functions as a plant growth promoter and bio-fertilizer in nutrient-deficient soils, imparts bio-protection against biotic and abiotic stresses, as a bio-regulator for plant growth development. Beneficial interaction are established for many plants of economic importance in agriculture, arboriculture, agro-forestry, floricultural including orchids, and those utilized for energy production and paper industry (Rai and Varma, 2005; Varma *et al.*, 2012). Studies have shown increased crop yield due to inoculation of AM fungi. Among several many important factors for large scale commercial production of *P. indica*, cost of synthetic media is of great importance (Baishya *et al.*, 2015). Synthetic media contain ingredients of known composition which can be duplicated with precision each time they are made and contain defined amounts of carbohydrates, nitrogen and vitamin sources (Basu *et al.*, 2015). Mass multiplication can easily be achieved in broth culture and bio-efficacy of inoculum produced is major concern (Ramanujam *et al.*, 2012). The
potential of the fungus as bio-inoculum can be fully exploited by use of cost effective and simple mass multiplication medium which could augment its usage for commercial purposes (Malus’a et al., 2012).

There exist many constraints to large scale commercial production of AM fungal inoculum production. Unlike AM fungi, P. indica can be easily cultivated on a variety of synthetic media (Baishya et al., 2015). Since P. indica is an important plant growth promoting agent having additional cultivation advantages unlike Vesicular Arbuscular Mycorrhiza, there is a need for some cost effective and simple mass multiplication medium which could augment its usage for commercial purposes. In order to achieve this aim, an attempt was made find a simple, cheap and easily available alternate medium which could be used as a replacement of high cost medium. Further, assessment of the bio efficiency of the fungus cultivated on different growth medium using Vigna radiata as a test crop was undertaken. The research was undertaken with two objectives: To assess the influence of the growth media on the quantity of fungal biomass generated and comparison of bio-efficacy of the fungal biomass generated in different growth media.

Materials and Methods

Fungal culture: Pure culture of fungus P. indica was obtained from School of Life Sciences, Jawaharlal Nehru University, (New Delhi), India. The culture was maintained on Hill and Kafer (2001) medium and stored at 4°C. Circular agar discs (5 mm diameter) having spores and actively growing hyphae were placed onto sterilized glass petriplates containing 20-25 ml solidified medium (Varma et al., 1999). Inoculated petriplates containing medium were incubated at 28°C in an upright position in dark for 7 days till the concentric growth ring pattern of fungal growth covered the entire plate.

Liquid cultures: Liquid cultures were grown on enriched soil medium, potato dextrose, Czapek Dox, Rose Bengal and malt extract medium (Hill and Kafer 2001). A series of 50-ml Erlenmeyer flasks containing 25 ml of above the media were each inoculated with 5 mm disc of P. indica and incubated statically at 30°C for 35 days. At weekly intervals following inoculation replicate flasks were destructively harvested for determination of residual sugars and mycelial dry weight.

Preparation of soil extract: 1000g soil was mixed with 1000 ml water and autoclaved at 121°C at 15 psi for 30 min. 0.5g l⁻¹ CaCO₃ was added before filtration and filtrate was used as soil extract. This extract was used to prepare enriched soil medium (containing 5.0g l⁻¹ Glucose; 0.5 g l⁻¹ K2HPO4; 0.5 g l⁻¹ Yeast Extract; 100 ml l⁻¹ Soil Extract; 900ml l⁻¹ Distilled water and 15g l⁻¹ Agar) for growth of P. indica.

Comparison of fungal biomass produced by various fungal media with enriched soil:

Extract medium: Different fungal media used included Kaefer's medium, Potato Dextrose Agar, Malt extract agar, Rose Bengal agar, Czapek Dox Agar and Enriched soil medium. These broth cultures were inoculated with agar discs having fungal biomass (CFU~28×10⁶ ml⁻¹) and incubated at 30 ± 2°C under static condition. Each treatment was replicated thrice. The fungal biomass produced was estimated in each treatment weekly for 5 weeks. The biological activity of the fungal biomass generated in different treatments was assessed in terms of efficiency of colonization of host plant, root phosphatases, rhizosphere soil phosphatases activity and plant growth parameters.

Testing bioefficacy of fungal inocula: A pot experiment was conducted using earthen pots (diameter 12cm), each having 300g unsterilized soil and kept under glass house condition. Seeds of Vigna radiata (PUSA 9072) were sown along with the fungal inoculum generated on six different agar growth media. Each treatment received equal inoculum per pot (2.0g on dry weight basis). Inoculum as discs of P. indica was applied as a cushion under the seed as per modified method of Varma et al. (1999). Pots without the inoculum served as control. After germination one plant pot was maintained. The growth conditions were 28±2°C, 16hr light 8hr dark with light intensity of 1000 Lux and relative humidity 70%. Plants were watered to 60% of water holding capacity.

Biochemical assays: Phosphatase activity was measured by the modified method of Tabatabai and Bremner, (1969) using 1g moist soil and 12 ml buffer-substrate per sample. Estimation of root colonization by P. indica was carried out by the method of Phillips and Hayman (1970).

Total root colonization and length of root colonized by hyphae, arbuscules and vesicles by AM fungi were estimated by modification of grid intersection method (McConigle et al., 1990) and slide method (Giovannetti and Mosse, 1980). Protein content of enzyme extract was estimated by Lowry's method (1951) using bovine serum albumin as standard. Chlorophyll content was determined by the method of Hiscox and Isrealstam, (1979).

Statistical analysis: All the data recorded were analyzed using the standard procedure of statistical analysis in a randomized block design (Gomez and Gomez, 1985). Data were subjected to Duncan’s Multiple Range Test (DMRT) for the analysis of significant difference between the treatments. Significance values were computed using ANOVA significance level of P<0.05. Data were analyzed using statistical package MSTATC.
Results and Discussion

Piriformospora indica inoculation was found to exert a positive influence on acid phosphatase activity in the rhizosphere of Vigna radiata. Enzyme activity showed a progressive increase with time in all the treatments. Significant variation in stimulation of enzyme activity following inoculation with the fungus cultivated on different growth media was recorded. The enzyme activity ranged from 76.41 μg PNP g−1 soil 24 hr−1 in control to 121.5 μg PNP g−1 soil 24 hr−1 in the Kaefer's broth media after 21 DAI (Table 1). Inoculation with fungus grown in Kaefer's medium increased enzyme activity by 59.01% over control, while in case of malt extract stimulation of enzyme activity was 40.95%. P. indica cultured in Kaefer's medium consistently supported highest acid phosphatase activity at three intervals (14, 21, 28 DAI) as compared to inoculum raised on other culture media. Fungus cultivated on Malt extract Medium exerted least positive effect on acid phosphatase activity in the rhizosphere of V. radiata. The results indicate that the chemical composition of growth medium used for inoculum production influenced the biological performance of inoculum.

The positive effect of fungal inoculation on alkaline phosphatase activity in the rhizosphere of V. radiata over control was recorded (Table 1). An increase up to 266% in the enzyme activity was recorded due to fungal inoculation as compared to control after 28 DAI. The beneficial effect of fungus raised on Kaefer’s medium was found to be identical and highest among the treatments. The magnitude of stimulation of enzyme activity following fungal inoculations ranged from 211.9% - 266% as compared to control. The order of stimulation of alkaline phosphatase activity as compared to control was in the following order KM=ESM>PDA>ME>Czapec Dox>RB.

Phosphatases represent a broad range of intracellular, as well as soil accumulated activities that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Maurya et al., 2011). Phosphatase enzymes are also directly involved in the acquisition of phosphorus by plants. Alkaline phosphatase has been ascribed to soil bacteria, as it is absent from the rhizosphere of plants grown axenically (Dodd et al., 1987). AM fungi also regulate root phosphatase exudation (Johri et al., 2015).

P. indica has been reported to enhance phosphatase activity (Varma et al., 2012). Thus, a consistently high activity was noted in P. indica grown on all media although the best results obtained were from Kaefer’s medium as well Enriched Soil Medium treatments. The observed differences induced by the culture medium in expressing the efficacy of fungus in stimulating phosphatase activity is similar to the observation of Amanullah et al. (2002) who reported that the conditions employed in the culture of biocontrol agent might significantly influence production of antimicrobial substances, it’s survival in soil and biological activity. The cultural conditions employed for the growth of Aspergillus oryzae plays a role in branching of the hyphae resulting in and thereby, regulates specific amyloglucosidase productivity in filamentous fungi. Hyphal branching leads to more tips, which leads to higher metabolic activity (Shoji et al., 2015). Thus, correlation exist between the number of tips and concentration of heterologous lysozyme produced by A. oryzae.

Table 1 : Acid and alkaline phosphatase activity recorded in the rhizospheric soil of Vigna radiata inoculated with Piriformospora indica cultivated on different growth media (μmoles pNPPg−1 soil)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>14 DAS</th>
<th>21 DAS</th>
<th>28 DAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid</td>
<td>Alkaline</td>
<td>Acid</td>
</tr>
<tr>
<td>Non inoculated control</td>
<td>51.43</td>
<td>10.57</td>
<td>61.59</td>
</tr>
<tr>
<td>Inoculum produced on Kaefer’s Medium</td>
<td>98.09</td>
<td>56.88</td>
<td>109.2</td>
</tr>
<tr>
<td>Inoculum produced on Enriched soil Medium</td>
<td>97.09</td>
<td>56.22</td>
<td>103.4</td>
</tr>
<tr>
<td>Inoculum produced on Potato Dextrose Medium</td>
<td>93.55</td>
<td>53.10</td>
<td>99.11</td>
</tr>
<tr>
<td>Inoculum produced on CzapekDox Medium</td>
<td>89.54</td>
<td>48.93</td>
<td>96.09</td>
</tr>
<tr>
<td>Inoculum produced on Malt Extract Medium</td>
<td>86.44</td>
<td>54.66</td>
<td>94.19</td>
</tr>
<tr>
<td>Inoculum produced on Rose Bengal Medium</td>
<td>86.44</td>
<td>54.66</td>
<td>102.2</td>
</tr>
<tr>
<td>CD at 5% Acid phosphatase</td>
<td>0.696</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD at 5% Alkaline phosphatase</td>
<td>0.674</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM Acid phosphatase</td>
<td>0.232</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM Alkaline phosphatase</td>
<td>0.224</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values followed by same letter are not significantly different according to Duncan’s multiple range test at p<0.05
Roots of moong bean (*Vigna radiata*) plants were used to estimate phosphatase activity after 28 days. The acid phosphatase activity of roots of inoculated plants was observed significantly higher as compared with control. The degree of promotion in activity was differential depending on the media used to culture *P. indica*. Highest activity was obtained in roots inoculated with *P. indica* cultivated in enriched soil medium and was closely followed by Kaefer's medium, potato dextrose agar medium, Czapek Dox medium and malt extract medium. Inoculation with *P. indica* cultured in enriched soil medium and Kaefer's medium increased root acid phosphatase activity by 104.9% and 97.2%, respectively, as compared to control. However, least (11.6%) stimulation of enzyme activity was found in roots inoculated with fungus cultured in rose bengal and malt extract medium. The alkaline phosphatase activity of roots of inoculated plants was found to be significantly higher as compared to control. The degree of promotion in enzyme activity was differential depending on the media used to culture *P. indica*. Malt extract medium raised inoculum yielded the highest activity (867.72%) followed by Kaefer's medium (774.69%), enriched soil medium (734.38%), potato dextrose (708.53%) and rose bengal (695.33%). However, least activity was obtained in Czapek Dox.

In the present study, the nutrient composition of fungal growth medium influenced root colonization, as well as, the acid and alkaline phosphatase activity. This observation is similar to Rizner and Romih (2007) who reported that the nutrient concentration of the medium influences colonization of plants by AM fungi. The possible reason for the above observation is the altered morphology of fungal hyphae and secretion of chemical factors that control the extent of root colonization. The composition of growth media is known to influence the metabolism, morphology and subsequent expression of fungal activity as reported by Ahamed and Vermette (2009).

Plant chlorophyll content assayed at 28 days after sowing of *V. radiata* seeds recorded an insignificant increase following inoculation with *P. indica* culture grown on different media (Table 2). The amount of chlorophyll synthesized in response to inoculations was lowest in rose bengal (2.772 mg plant$^{-1}$) and highest in enriched soil medium (3.674 mg plant$^{-1}$). Hence, the chlorophyll content was not effectively enhanced in inoculated treatment as compared to control. The chlorophyll content was not significantly influenced by fungal inoculation and was comparable to control. Inoculation failed to promote chlorophyll synthesis in any of the treatments.

The results on nitrogen uptake (Table 2) by plant tissue indicated highest nitrogen content (19.260 mg plant$^{-1}$) with *P. indica* cultured on Kaefer's medium followed by enriched soil medium (19.123 mg plant$^{-1}$), malt extract medium and Czapek Dox. The least promotional activity was obtained in case of *P. indica* cultured on rose bengal. All the inoculated treatments, however, showed increased nitrogen uptake as compared to control (9.077 mg plant$^{-1}$). Nitrogen content in *V. radiata* plants estimated after 28 days was enhanced by *P. indica* inoculation. This observation was similar to that of Sherameti *et al.* (2005).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf chlorophyll content (mg g$^{-1}$ d. wt.)</th>
<th>Root acid phosphatase activity (umoles-NPP g$^{-1}$)</th>
<th>Root alkaline phosphatase activity (umoles-NPP g$^{-1}$)</th>
<th>Nitrogen uptake (mg plant$^{-1}$)</th>
<th>Phosphorus content (mg plant$^{-1}$)</th>
<th>Dry Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non inoculated control</td>
<td>3.545a</td>
<td>34.00</td>
<td>3.833a</td>
<td>9.08a</td>
<td>6.36a</td>
<td>1.69a</td>
</tr>
<tr>
<td>Inoculum produced on Kaefer’s medium</td>
<td>3.34a</td>
<td>67.05</td>
<td>33.52a</td>
<td>19.26a</td>
<td>17.02a</td>
<td>3.42a</td>
</tr>
<tr>
<td>Inoculum produced on Enriched soil medium</td>
<td>3.69a</td>
<td>69.69</td>
<td>31.98a</td>
<td>19.12a</td>
<td>17.15a</td>
<td>3.35a</td>
</tr>
<tr>
<td>Inoculum produced on Potato dextrose medium</td>
<td>3.367a</td>
<td>46.34</td>
<td>30.99a</td>
<td>18.72a</td>
<td>17.09a</td>
<td>3.24a</td>
</tr>
<tr>
<td>Inoculum produced on Czapek Dox medium</td>
<td>3.162a</td>
<td>42.38</td>
<td>25.358a</td>
<td>17.71a</td>
<td>16.44a</td>
<td>2.66a</td>
</tr>
<tr>
<td>Inoculum produced on Malt extract medium</td>
<td>3.412a</td>
<td>37.97</td>
<td>37.09a</td>
<td>18.19a</td>
<td>16.74a</td>
<td>3.16a</td>
</tr>
<tr>
<td>Inoculum produced on Rose Bengal medium</td>
<td>2.77a</td>
<td>37.97</td>
<td>30.49a</td>
<td>17.39a</td>
<td>14.43a</td>
<td>3.11a</td>
</tr>
<tr>
<td>CD at 5%</td>
<td>0.453</td>
<td>1.337</td>
<td>2.910</td>
<td>1.207</td>
<td>1.811</td>
<td>0.519</td>
</tr>
<tr>
<td>SEM</td>
<td>0.1494</td>
<td>0.440</td>
<td>0.953</td>
<td>0.398</td>
<td>0.596</td>
<td>0.1713</td>
</tr>
</tbody>
</table>

*Values followed by same letter are not significantly different according to Duncan’s multiple range test at p<0.05*
The fungal biomass (dry weight) obtained on different growth media at weekly interval is presented in Table 2. Initially, 7 days after inoculation (DAI) dry weight was highest in Kaefer’s medium (50mg). After an incubation period of two week maximum biomass (94 mg 50ml⁻¹) was recorded in enriched soil medium followed by Kaefer’s medium (89 mg 50ml⁻¹) and it was least in Czapek Dox (31 mg 50ml⁻¹). In terms of their ability to support fungal growth at 14 DAI, different media were found in the following order: Enriched Soil Medium> KM> PDA> ME> RB. After 21 days and up to 35 days, maximum mycelial growth was recorded in Kaefer's Broth. The fungal biomass reached a plateau after 3 week incubation period in the enriched soil medium, while in potato dextrose and Kaefer's medium the fungal growth continued to increase up to 28 days. Of the six culture media tested, Malt extract medium and the rose bengal supported least fungal growth. At 35 DAI, PDA and Enriched Soil Medium supported an identical fungal growth.

The present study revealed that P. indica has potency to grow axenically on number of complex and synthetic media (Pham et al., 2004). High biomass generated in enriched soil medium and Kaefer's medium might be due to improved C: N ratio. The C: N ratio was reported to have significant effect on growth of fungus (Moh et al., 2013). Influence of culture media and and environmental factors on mycelial growth and sporulation of Lasiodiplodia theobromae were also reported by Saha et al. (2008).

A significant but variable increase in plant dry weight following fungal inoculation was observed after a growth period of 28 days (Table 2). Maximum dry weight of plants was observed by inoculation with fungus cultured Kaefer's medium (3.416 g pot⁻¹) and enriched soil (3.349 g pot⁻¹). Least plant dry weight was recorded by inoculation with fungus grown in czapex dox (2.660 g pot⁻¹) but it was still higher as compared to control (1.685 g pot⁻¹). Overall, fungus inoculation significantly increased plant dry weight content, and the growth medium used for fungus production appeared to play a role in the expression of beneficial effect.

The dry weights of plants taken after 28 days showed positive effect of fungal inoculation. These results are similar to the earlier reports indicating the fungus mediated enhancement of plant biomass (Meena et al., 2010). The maximum amount of weight was obtained in Kaefer’s treatment closely followed by enriched soil medium. These results confirm that enriched soil medium is as good as Kaefer’s medium as compared to other media currently being used for culturing of P. indica and hence, can be used as an alternate low cost medium for mass production, as well as for general laboratory purposes.

The plant growth promoting microorganisms promotes circulation of nutrition and reduces the need of chemical fertilizer. Kafer medium and enriched soil medium found to be simple, cheap and easily available alternate medium which could be replaced by high cost medium, currently under use for its multiplication.

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**References**


