

Impact of Culture Filtrate of *Piriformospora indica* on Biomass and Biosynthesis of Active Ingredient Aristolochic Acid in *Aristolochia elegans* Mart

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Abstract

The mycorrhiza plant partnership is the basic, essential and integral part of plant survival and growth. In the present investigation we are reporting the effect of culture filtrate of *Piriformospora indica*, a growth promoter and bioprotector fungus on *Aristolochia elegans* Mart. The culture filtrate of the fungus increased overall growth, biomass, and active ingredient-aristolochic acid in the leaves of plants. In untreated control plants, the overall growth was reduced. *P. indica* culture filtrate application increased root number, root length, and root dry weight by 28%, 98%, and 123% respectively in plants of *Aristolochia*. Also stem height and shoot length was enhanced by 43% and 155% respectively. There was increase in number of leaves by 79% and length of leaves by 36%. The increase in total biomass was 136%. The improvement in content of aristolochic acid in leaves was between 7.6% and 28.8% in treated plants as against untreated control plants

Keywords: *Aristolochia elegans*, aristolochic acid, *Piriformospora indica*, symbiotic fungus

1. Introduction

The fungus *Piriformospora indica*, is related to the Hymenomycetes of the Basidiomycota, which is a root endophyte that has capabilities of a typical, arbuscular mycorrhizal fungus, (Verma et al., 1998; Weiss et al., 2004; Prasad et al., 2008a; Bagde et al., 2010a, 2010b, 2010c, 2011), but unlike Arbuscular Mycorrhizal fungi it is cultivable in axenic condition easily. This mycorrhiza like fungus can form association with roots for enhanced growth and development of plants (Varma et al., 1999, 2001; Oelmüller et al., 2009; Sirrenberg et al., 2007; Prasad et al., 2008a; Bagde et al., 2011). *P. indica* interacted mutually with various plants including *Fabaceae* and *Rhamnaceae* species (Varma et al., 2001), *Arabidopsis* (Peškan-Berghöfer et al., 2004), tobacco (Barazani et al., 2005), *Poaceae* species (Waller et al., 2005).

P. indica enhanced nutrient uptake, helped plants to survive in extreme drought, temperature and salt conditions, exhibited systemic resistance to toxins, acted as biofertilizer, bioprotector, stimulator of growth, increased seed production, and played a key role in increasing the tolerance to insects (Varma et al., 1999; Waller et al., 2005, 2007; Serfling et al., 2007; Prasad et al., 2008a, 2008b). It helps in biological hardening to tissue culture raised plants, provides protection against 'shock of transplantation' and pathogens of roots (Sahay & Varma, 1999; Hazarika, 2003; Prasad et al., 2008a, 2008b).

Aristolochia elegans plant belongs to Aristolochiaceae family. It is generally called pine vines or Dutchman's pipes. It is an annual plant with slender, woody stems, climber on support with heart shaped leaves and calico flower. It grows all over the world including in tropical climates such as India and other countries and is also called birthworts and commonly used to ease pain of childbirth, treat malaria and other diseases (Kimura & Kimura, 1981). Plant is cultivated and used in some medicinal preparations in China (Lopes et al., 2001). It contains important alkaloid aristolochic acid which is antimicrobial in nature (Imran & Bagde, 2007) and is useful for variety of ailments. The fruits and roots have been used by Chinese people in medicine as anodynes, antiphlogistics, expectorants and anti-asthmatic agents and is also used in treatment of snakebite, anti-tumor, anti-platelet aggregator agent and lung inflammation (Vila et al., 1997; Wu et al., 1999; Tian-Shung et al., 2000).

However, its certain harmful activities such as mutagenicity and carcinogenicity have also been reported (Arlt et al., 2002).

So far all the accounts are on the interaction of fungus proglules but in this communication we document that *P. indica* culture filtrate also enhanced the overall growth parameters of *Aristolochia elegans* and contents of aristolochic acid in leaves.

2. Materials and Methods

2.1 Mycobiont

P. indica culture for this study was procured from Amity University's Amity Institute of Microbial Technology, India.

2.2 Photosymbiont

Aristolochia elegans Mart. (Aristolochiaceae) is the perennial shrub cultivated as ornamental plant in India. Species of *Aristolochia* are cultivated and used in medicinal preparations (Lopes et al., 2001). The plantlets were procured from Jijamata Udyan Byculla, Mumbai, India and were multiplied in environmentally controlled green house. Sterile substratum was used to conduct the experiments.

2.2.1 Culturing the Fungus *P. indica*

A. elegans was cultivated and maintained on modified synthetic media fortified with 1.2% agar (w/v) in dark at 28 ± 2 °C (Hill & Käfer, 2001; Prasad et al., 2005). pH of medium was kept at 6.5. For mass propagation, the fungus was also cultivated in liquid broth medium under constant shaking at 120 rpm in dark (GFL 3019, Germany). Media were sterilized in autoclave at 15 psi pressure for 15 minutes.

2.2.2 Separation of Culture Filtrate

The fungus was grown in liquid medium for 15 days and was first filtered through sterile muslin cloth followed by bacterial filter (Millex-GV, 0.22 µm Filter Unit, Millipore) and kept at 4 °C if not used afresh.

2.2.3 Co-Cultivation Experiments

Plantlets grown for Fifteen days were transferred to sterile 10" diameter plastic pots containing sterile unfertilized garden soil autoclaved on three consecutive days. Initially two plantlets were planted in each pot. Once they got acclimatized then one of the plantlet was removed, finally retaining only one plantlet in each pot. To each pot containing 1 kg of soil 15ml of freshly eluted culture filtrate to experimental pots and an equal volume of sterile nutrient medium were added to control pots one day before transfer of the plantlet into the pots. Again after a period of one month this treatment was repeated.

2.2.4 Growth Conditions

Pots were kept in green house at temperature of 26 ± 2 °C and 16 h light/8h dark and 60%-70% relative humidity and a light intensity of 20,000 lux. Growth of plants was measured after 90 days by use of centimeter scale. For estimation of dry biomass, plant was chopped and dried at 80 °C for 12 h in a Memmert oven and dry biomass was estimated after cooling at room temperature and weighing on electric- mono-pan balance.

2.3 Aristolochic Acid Analysis

Leaves of *Aristolochia* were used to extract and estimate aristolochic acid. For preparation of extract, leaf material was ground to fine powder by mechanical grinding using HPLC grade methanol and formic acid. 2 gm. of ground sample was taken in a bottle, thoroughly mixed with a mixture of 50 ml of methanol (80%) and 20 ml of 10% formic acid in water. The contents were stirred for 30 minutes at 500 rpm (Innova Model 2001 bench top platform shaker, New Brunswick, USA) and then centrifuged for 4 minutes at 4000 rpm. The supernatant was taken for determination of aristolochic acid (Gaudreault et al., 2001; Flurer et al., 2001). Estimation of aristolochic acid in the leaves extract of *A. elegans* was carried out by HPTLC using standard aristolochic acid as reference (Sigma, USA) in the range of 0-200 µg/ml. Stationary phase used for HPTLC contained Silica gel 60 (Merck) plates of 10x10 cm size. The mobile phase used for the chromatogram consisted of toluene, ethyl acetate, water and formic acid in the ratio of 20:10:1:1. The sample used was 10 µg. For developing the plate twin trough chamber was saturated for 20 minutes and the plate was dried with hair drier (cold air) for 5 minutes. The plate was evenly sprayed with tin (II) chloride reagent and further dried at 100 °C for a minute. Plates were observed under UV light at 366 nm and acid content was measured. This was determined after 15, 30, 45, 60, 75 and 90 days.

3. Results and Discussion

When morphological appearance of *P. indica* was observed on Käfer agar medium the pattern of growth of the fungus was marked by uniform rhythmic zonation (Figure 1a). The rapid growth on Käfer nutrient broth was observed after 15 days incubation at temperature of 28 ± 2 °C (Figure 1b). The colonies showed prominent crowded balls of coral morphology in conformity with previous studies by various workers (Varma et al., 2001; Singh et al., 2003). The important characteristics of this organism have been described earlier (Varma et al., 2001).



Figure 1(a). Growth of *P. indica* on solidified agar medium

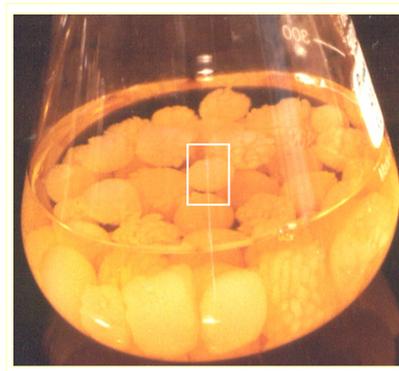


Figure 1(b). Cultivation of *P. indica* in aspergillus broth medium

The observations made in this study indicated that culture filtrate of fungus exerted positive impact on various parameters of the plant as depicted in Figures 2 & 3 and Tables 1 & 2. When *A. elegans* plant was treated with culture filtrate *P. indica*, it enhanced the number, length and biomass of the root (Table 1). Pretreatment also resulted in an increase in root number, root length and root dry weight by 28%, 98%, and 123% respectively in *Aristolochia*. Increased root length and number can enhance absorption of more nutrients due to increased absorbing area resulting in improved plant growth (Marschner & Dell, 1994). Similar observations were made by other workers (Mugnier & Mosse, 1987; Varma et al., 2001). Inoculation of culture filtrate in case of grasses, trees and herbaceous sp. also showed enhancement of plant growth (Varma et al., 2001).



Figure 2. Effects of *P. indica* culture filtrate inoculation on *Aristolochia elegans*(A treated and B untreated)

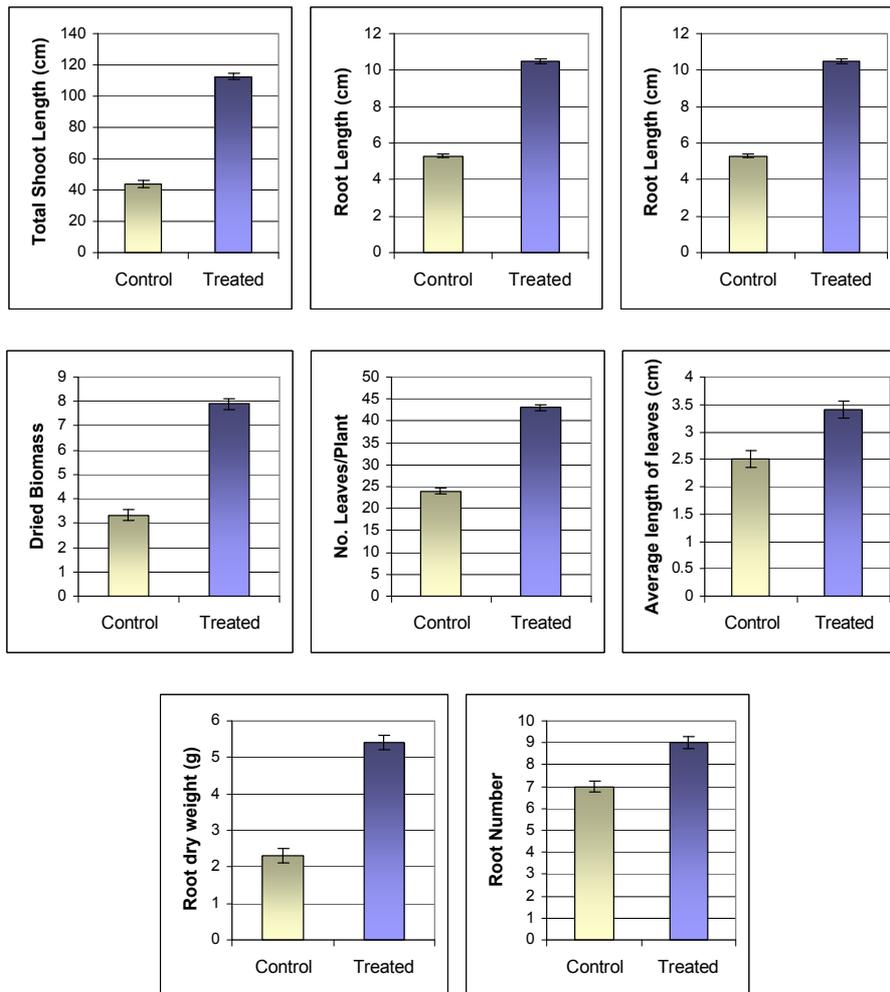


Figure 3. Effect of *P. indica* culture filtrate on *Aristolochia elegans*

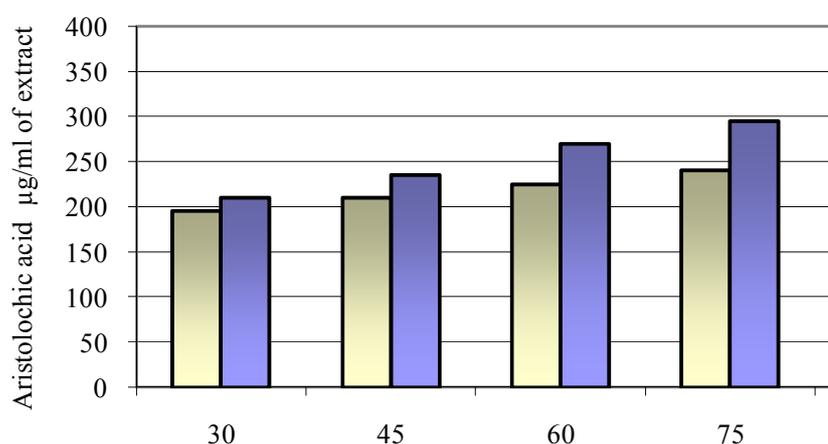


Figure 4. Effects of *P. indica* inoculation on Concentrations of Aristolochic acid in leaves of *Aristolochia elegans*

Table 1. Effect of *P. indica* culture filtrate on growth performance of *Aristolochia elegans*. Average values are for five replicates

Characteristics		Control (untreated)	Experimental (<i>P. indica</i> treated)	S. E.	S. D.	Percent increase over control
Root	Number	7.00	9.00	± 0.26	0.2	28
	Length(cm)	5.30	10.5	± 0.12	0.12	98
	Dry weight (g)	2.30	5.4	± 0.20	0.20	123
Stem	Height(cm)	7.2 0	10.5	± 0.02	0.02	43
	Shoot length(cm)	44.00	112.5	± 2.17	2.17	155
Leaves	Number	24.00	43.0	± 0.68	0.68	79
	Length(cm)	2.50	3.4	± 0.15	0.15	36
Total Biomass (g)	Roots, Stems, Leaves,	3.32	7.9	± 0.22	0.22	136

S. E = Standard Error; S. D. = Standard Deviation.

Table 2. Effects of *P. indica* inoculation on Concentrations of Aristolochic acid in leaves of *Aristolochia elegans*

Days After planting	Aristolochic acid µg/g of extract		S. D.	Percent increase over the Control
	Un-treated (Control)	Treatment with <i>P. indica</i>		
30	195 ± 1.76	210 ± 1.76	1.76	7.6
45	210 ± 2.10	235 ± 2.10	2.10	11.9
60	225 ± 5.64	270 ± 5.64	5.64	20.0
75	240 ± 4.85	295 ± 4.85	4.85	22.8
90	260 ± 6.47	335 ± 6.47	6.47	28.8

Enhanced root growth, and root length was observed after application of the fungus in several plant species studied earlier (Varma et al., 2001). Not only the mycelium but even culture filtrate enhanced growth of the plants. Earlier it was reported that plant root cells can be killed by colonization of this fungus (Deshmukh et al., 2006), however it also increased root growth, weight and branching (Varma et al., 1999; Waller et al., 2005).

Increased rooting of calli of *N. tabacum* and cuttings of other plants was also noticed (Varma et al., 1999; Drudge et al., 2007). When culture filtrate of *P. indica* was applied, a diffusible factor from it enhanced root growth of *Arabidopsis*. There was stunted but highly branched roots in treated plants (Sirrenberg, 2007). The overall increment in the plant growth reported in this study may be due to increased nutrients uptake by the roots. This may also be due to application of culture filtrate that contained many growth promoters that exerted desirable effect on plant.

When culture filtrate of *P. indica* was applied, Increments in stem height and shoot length of plants were observed in plants in the present study (Table 1). Stem height increased by 43% and shoot length by 155%. This is in conformity to observations made in earlier investigations (Varma et al., 2001; Nautiyal et al., 2010; Bagde et al., 2010a, 2011).

The number of leaves increased by 79% and length by 36% in *P. indica* culture filtrate treated plants in comparison to untreated plants (Table 1) Increase in number and length of leaves was also reported in other plants using fungal mass or fungal culture filtrate (Varma et al., 2001; Fakhro et al., 2010; Bagde et al., 2011). A greater number of leaves, with increased length produced in treated plants could have contributed to increased rate of photosynthesis (Kungu, 2004).

P. indica culture filtrate treatment enhanced growth as well as total biomass of plants in comparison to untreated control plants in present study (Table 1). Similarly there was reported increase in total biomass by 136% as against treated control plants in herbaceous species (Varma et al., 2001) and *Helianthus annus* (Bagde et al., 2011), winter wheat plants (Serfling et al., 2007).

When six strains of *Sebacina vermifera* were tested on *Panicum virgatum* roots, it was noticed that there was positive effects on plant height and biomass production. It was also observed that culture filtrates from some strains of *S. vermifera* increased seed germination in *P. virgatum* by 52% over the control. In spring barley *P. indica* increased plant biomass and grain yield by 11% (Waller et al., 2005). Serfling et al. (2007) observed that fungus *P. Indica* colonization increased plant biomass in winter wheat plant.

When fungal culture filtrate was applied to the soil before planting, it increased total content of aristolochic acid in leaves between 7.6% to 28.8% (Table 2). The quantity of leaves and content therein were augmented as compared to control plants when treated plantlets were transferred to the pots. This positive influence in promoting the plant growth and yield in terms of biomass and medicinal ingredients may be due to positive effect of stimulatory factors or components present in the culture filtrate.

Besides several reports pertaining to the association of cells of *P. indica* with plants that enhanced growth, present study reports positive effect of even culture filtrate of fungus on plant growth. This is due to special characteristics of culture filtrate that was used. Culture filtrate is a complex growth enhancer of which all ingredients are not known (Bagde et al., 2010b). Culture filtrate contains fungal exudates, hormones, enzymes, proteins etc. that increased root number, length, root dry weight, stem height, shoot length, number and length of leaves, total biomass and aristolochic acid content of leaves in culture filtrate treated plants. Similar observations were made in case of maize, *Bacopa monniera*, and tobacco (Varma et al., 2001), neem and maize (Kumari, 2002; Singh et al., 2003). In *Helianthus annus*, treatment with *P. indica* culture filtrate promoted overall growth of the plant in terms of increased, root collar diameter, number of secondary roots, root length, root weight, stem diameter, stem height, number of leaves, length and width of leaf, flower number, flower diameter, flower dry weight, number of seeds, weight of seeds and total biomass as compared to untreated control plants. Seed oil content considerably increased in treated plants. Seed oil content increased by 51.13 per cent in sun gold variety and 70.33 per cent in treated Japanese gold variety of *H. annus* plants (Bagde et al., 2011).

Varma et al. (2001) also reported that application of culture filtrate of *P. indica* led to increase in root length, shoot length and plant biomass in treated plants. In present study treatment of *Aristolochia elegans* increased growth of roots, stems, leaves, total biomass as well as aristolochic acid over untreated plants (Table 1). These observations are in conformity to observations of Singh et al. (2003) wherein treatment resulted in considerable increase in growth and development in *Azadiracta indica* and *Zea mays* plants. Similarly when *Helianthus annus* plants were treated with culture filtrate of *P. indica*, root number, length, root collar diameter and dry weight of root increased considerably (Bagde et al., 2011). Observations like these were also made by other investigators, who reported luxurious and elaborate root growth and biomass when treated with mycelia of fungus (Varma et al., 2001; Kungu, 2004).

According to Sirrenberg et al. (2007) actual mode of action of *P. indica* in enhancing the growth of plants was not yet clear. But it is suggested that effect was due to diffusible factor that could be IAA, as *P. indica* was found to produce IAA in culture filtrate in sufficient quantities and hence it must have contributed to the beneficial

effect on its host plants. The fungus may in addition induce auxin production in the plant (Peškan-Berghöfer et al., 2004). Plants colonized with *P. indica* can tolerate physical stress, nutrient deficiency, biotic and abiotic stresses and can fight pathogens including invaders of insects and facilitated increase in seeds and early flowering in medicinal plants (Oelmüller et al., 2009).

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