

# In vitro co-cultivation of *Piriformospora indica* filtrate for improve biomass productivity in *Artemisia annua*(L.)

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**Abstract** We established in vitro augmentation of callus, organogenesis and photosynthetic pigments content in *Artemisia annua* using *Piriformospora indica* filtrate and by optimizing plant growth regulators on culture medium. In vitro raised plantlets from nodal explants showed the best shooting at MS + BAP (3 mg/l) while ½ MS + IBA (3 mg/l) was the best combination for rooting. Co-cultivation of nodal explants using *P. indica* was done just after root initiation. Leaf explants supplemented with MS + 2, 4 D (3 mg/l) showed the best callogenesis. Interaction of in vitro raised plantlets and callus with the fungus and the filtrate resulted in overall increase in plant biomass and chlorophyll contents. Within 15 days of inoculation, the fungus colonized with the root cortical region of the plantlet and showed an increase in growth parameters such as root (21 %) and shoot (37.2 %) length, fresh (35 %) and dry weight (3 %), and most importantly pigment contents i.e. chlorophyll a (2 %), chlorophyll b (0.3 %) and carotenoid (66 %). *P. indica* filtrate (1 µl/ml)-treated light green friable callus obtained from leaf explants using MS + 2,4 D (3 mg/l), showed the highest biomass within 35 days. Thus, the study demonstrated the symbiotic effect of *P. indica* as a bioprimer agent for the overall growth of biomass in *Artemisia annua*.

**Key message:** Co-cultivation of the *P. indica* culture filtrate with *Artemisia annua* can increase the growth and development of the plant which may have biotechnological application

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**Keywords** *Artemisia annua* · In vitro culture · *Piriformospora indica* filtrate · Root endophytes · Biomass productivity

## Abbreviation

BAP 6-Benzylaminopurine  
Kn Kinetin  
IBA Indole butyric acid  
MS Murashige and Shoog  
µl/l micro litre/litre

## 1 Introduction

*Piriformospora indica*, a member of the Sebaciales from genus Agarimycotina, Basidiomycota (Weiss et al. 2004) was isolated in the Indian Thar desert in 1997 (Verma et al. 1998). The fungus 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, b, c, 2011), but unlike Arbuscular Mycorrhizal fungi it is cultivable in axenic conditions easily; it can be cultured on various synthetic, simple and complex media within the temperature range 25–35 °C (Varma et al. 1999). The fact that the fungus interacts with *Arabidopsis thaliana* facilitated to explore the mechanisms of the interaction between *P. indica* and plants (Peskan-Berghofer et al. 2004). It has been reported as effective new candidate symbiont for providing enormous growth-promoting activity to a wide range of plants species, including agricultural and medicinal crops (Tsimilli-Michael and Strasser 2013); Monocots, eudicots, and dicots (Verma et al. 1998; Pham et al. 2004a, b; Shahollari et al. 2007; Varma et al. 1999; Varma et al. 2001 and Waller et al. 2005).

It has been reported to help in acclimatization of tissue culture raised plants, provides protection against shock of transplantation and pathogens of roots (Sahay and Varma 1999; Hazarika 2003; Prasad et al. 2008a, b). *P. indica* improves the plant growth, nutrient uptake, and helped plants during abiotic and biotic stress tolerance. Because of these characters, *P. indica* has proven to be a plant biofertilizer, probiotic, and biohardening tool (Sun et al. 2010; Stein et al. 2008). The growth promotion effect of *P. indica* has been reported in the medicinal plants *Adhatoda vasica* (Rai and Varma 2005) and *Centrella asiatica* (Satheesan et al. 2012). Baldi et al. (2010) reported on enhanced production of podophyllotoxin in cell suspension cultures of *Linum album* by elicitation with culture filtrate and cell extract of *P. indica*. Realizing the growth promoting activity of *P. indica* upon colonization with the roots, an attempt was made to study the impact of *P. indica* filtrate on overall biomass production in the in vitro grown *A. annua* plantlets. The impact of *P. indica* on *A. annua* callus growth index is studied for the first time.

*Artemisia annua*, belonging to the family Asteraceae is native to China, locally known as qinghao (green herb) and has been used for over 2000 years to treat symptoms associated with fever and malaria. It is known in the United States as sweet Annie, annual or sweet wormwood (Ferreira and Janick 1997). It is a shrub, often attained a height of over 2 m. Its leaves and flowers contain artemisinin, an antimalarial drug, first isolated in China in 1971 (Ferreira and Janick 1997). However, the production of artemisinin although can be increased through larger scale field cultivation, the length of cultivation and manufacturing time, the need for a large area of land and labour, and the expense of extraction are major hindrances. Hence, alternative approaches are being adopted to enhance artemisinin production using in vitro methods (Wang et al. 2006). Therefore, we have adopted an in vitro culture technique, as it is possible to increase the number of the plants and to gainfully manufacture the bioactive principles through this technique.

Recently, micropropagation and organogenesis of different *Artemisia* species such as *A. mutellina* (Mazzetti and Donata 1998), *A. scorpioides* (Aslam et al. 2006), *A. vulgaris* (Govindaraj et al. 2008), *A. absinthium* (Zia et al. 2007) have been established by using several parts of the plant. Similarly, regeneration and multiple bud induction; using leaf, shoot tip, young florescence segment has been developed in *Artemisia annua*. Moreover, in vitro direct organogenesis of different parts of *Artemisia annua* to obtain large number of plant true type, multiplication of selected clones with high level of secondary metabolite and utilization of this protocol in future genetic transformation has been reported.

Considering the medicinal use of *Artemisia annua* and understanding the global interest towards eradication of malaria, there is an urgent need to enhance the biomass productivity of the plant. Moreover, *P. indica* has been reported to enhance

biomass productivity of the host plant upon co-cultivation (Verma et al. 1998). Therefore, our experiments aimed at enhanced production of artemisinin in in vitro raised *A. annua* treated with *P. indica*. In the present investigation, we have reported the influence of *P. indica* filtrate on biomass productivity in *A. annua*.

## 2 Materials and method

### 2.1 Plant material and explant preparation

Actively growing healthy stems of *Artemisia annua* having a diameter of 2–3 mm and leaves were harvested during the pre-flowering stage in the month of May from the Gauhati University campus, Assam, India. The plant material after harvest were brought to the laboratory immediately and processed for sterilization. Thoroughly washed explants were cut into small pieces near the internode region and treated with Tween 20 (5 % w/v) for 5 min. Surface sterilization was carried out by dipping the explants in 0.1 % HgCl<sub>2</sub> for 3 min followed by 3–4 rinses in sterilized double distilled water aseptically.

### 2.2 Culture medium

MS (Murashige and Skoog 1962) medium solidified with 0.8 % agar was used as a substratum for inoculation of explants. The pH of the medium was adjusted to 5.8. The culture was incubated at a control temperature of 25 ± 2 °C with 14 h photoperiod (3000 lx) and 10 h darkness.

### 2.3 Cultivation of *P. indica*

The *Piriformospora indica* DSM 211827 was provided by Prof. Ajit Varma, Amity University, Noida, India. Unlike the arbuscular mycorrhizal fungus as *P. indica* can be grown in various synthetic, simple and complex medium. Moreover, the effective co-culture does not depend on the pre-culture condition of the fungus. Therefore, the fungus was cultivated in PDB (Potato Dextrose Broth) medium under the optimized cultural conditions with agitation speed: 200 rpm; initial pH: 6.5; temperature: 30 °C; in 500-ml Erlenmeyer flask. The culture was centrifuged after 10 days and, filtered through Whatmann filter paper No.1 with a pore size of 11 µm. As the fungus have been reported to contain associated bacteria, the filtrate was further filtered through bacterial filter (Millex-GV, 0.22 µm Filter Unit, Millipore) and kept at 4 °C until used. In this condition, the filtrate remained active for one month.

## 2.4 Shoot initiation

Nodal explants were cultured on MS (Murashige and Skoog 1962) medium supplemented with different concentration of cytokines, Benzylaminopurine (BAP) and Kinetin (Kn) for shoot initiation while the basal medium without cytokines was used as control.

## 2.5 Root initiation

Shoots obtained from the nodal explant was inoculated on full MS and ½ MS medium supplemented with various concentration of IBA (1 mg/l, 2 mg/l and 3 mg/l) to observe the induction of root.

## 2.6 Establishment of callus culture

Leaves from 2<sup>nd</sup> and 3<sup>rd</sup> node of *Artemisia annua* were used as explants for induction of callus. Aseptically excised leaf pieces ( $\approx 1.2 \text{ cm} \times 1.2 \text{ cm}$ ) were cultured in glass culture vessels (250 ml) containing 75 ml of MS medium supplemented with 2, 4-D (3 mg/l). Using a digital weighing scale, fresh and dry weight of the callus of *A. annua* was measured at regular intervals. The dry weight of callus was obtained by air-drying fresh callus at room temperature until a constant weight was attained. The morphology and colour of the callus were also observed and recorded. The specimens of the callus tissue were weighed and the growth index was determined according to the following formula (Prashanth et al. 2012).

$$\text{Growth Index (GI)} = \frac{\text{Final weight of Biomass}}{\text{Initial weight of Biomass}}$$

## 2.7 *P. indica* culture filtrate treatment

The role of *P. indica* on root initiation and elongation was studied. For the purpose, two experiments containing equal number of microshoots (30 each) were taken. In one set up *P. indica* filtrate (1  $\mu\text{l}$ ) was added into the medium during the time of inoculation of each microshoot in ½ MS+ IBA (1, 2 and 3 mg/l). In the other, *P. indica* filtrate was added just after the initiation of roots. *P. indica* filtrate was also used to observe the biomass productivity in callus. For this, 4  $\mu\text{l}$  of the culture filtrate was added after initiation of callus.

## 2.8 Hardening

In vitro raised plantlets with well developed roots were removed from the culture medium, washed gently with running tap water to remove the agar and then transferred to plastic pots containing autoclaved soil, vermicompost (10:1). Plants were covered with transparent polyethylene bags to maintain

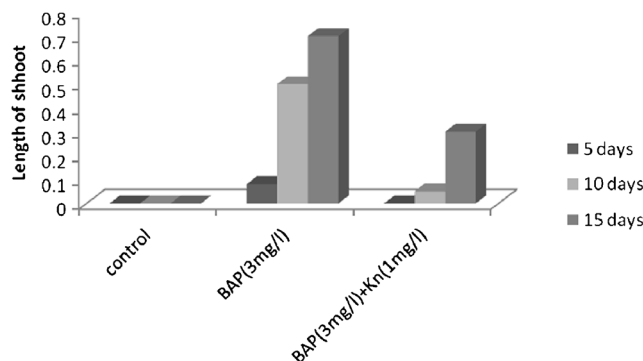
adequate moisture content for a week and transferred to green house (18 °C day, 20 °C night, 16 h day length, 70 % relative humidity). The plants were treated with ½ MS media for first 1 week. Regular watering of the plant was done on 2<sup>nd</sup> week. In the 3<sup>rd</sup> week, the plants were transferred to field.

## 2.9 2.8 *P. indica* Co-cultivation

For co-cultivation of rooted plantlets of *A. annua* with *P. indica*, fungal inoculation was provided during in vitro hardening of the plantlets. For this purpose, plantlets were divided into three groups containing 20 plantlets in each. The first group was considered as control. In the second group, mycelium was harvested by simply draining the culture medium from broth cultures leaving the fungus in the flask which was washed several times with sterile double distilled water sieving the fungus through double-lined cheese cloth each time. Mycelium about 50 mg fresh weight per plant was mixed with autoclaved soil. Prior to mixing the mycelium was mechanically homogenized in sterile distilled water in the ratio of 1:1 (w/v). This mycelium suspension was used to the roots by dipping them in the suspension. By this, the roots were layered with the mycelial suspension. It provided inoculum both on the root surface and in soil. The third group received the autoclaved fungal mycelium.

## 2.10 2.9 *P. indica* colonization

Ten rooted plantlets of *Artemisia annua* from control, treated with autoclaved *P. indica* and live *P. indica* were randomly selected after 10 days of co-cultivation in the green house condition. Colonization of *P. indica* was checked under the microscope (Lawrence & Mayo, Model NLCD-307; Halogen Lamp 6 V 20 W; No-000118; Operating System-WinCE-5.0). For the purpose, samples were softened in 10 % KOH solution for 15 min, acidified with 1 M HCl for 10 min, and finally stained with 0.02 % Trypan blue overnight



**Fig. 1** Nodal explants of *Artemisia annua* treated with cytokines (BAP and Kn) showing shooting response on 5th, 10th and 15th day of inoculation

**Table 1** Shoot multiplication after 30 days of in vitro culture of nodal explants of *A. annua* cultured in MS medium supplemented with different concentration of BAP. The values represents the means of 5 independent measurements  $\pm$ S.E; all data are found to be significant at  $p < 0.05$

BAP(mg/l) concentration	Number of shoots per explant Mean number of shoots $\pm$ S.E
Control	1.2 $\pm$ 0.27
2.5	6.3 $\pm$ 0.29
3	10.5 $\pm$ 0.37
3.5	5.7 $\pm$ 0.36

(Kumar et al. 2009; Dickson and Smith 1998). Samples were distilled with 50 % lacto-phenol for 1–2 h prior to observation under the light microscope. The distribution of intracellular chlamydo spores within the cortex region of root was taken as an indication of colonization (Varma et al. 1999).

### 3 Growth parameters of plantlets

Plant growth parameters such as root length, shoot length, dry weight and fresh weight were recorded and compared among plants containing treatment (control), treated with *P. indica* culture filtrate and autoclaved *P. indica*.

#### 3.1 Photosynthetic pigment analysis

The chlorophyll a, b and carotenoids content of in vitro grown *A. annua* plantlets was determined. Leaves were harvested, weighed, and ground in 90 % ammoniacal acetone (acetone: water: 0.1 N ammonia, ratio of 90: 9: 1) at 4 °C. The supernatants were used to measure absorbance at the following wavelength; 663, 645, and 470 nm for Chl a, Chl b, and carotenoids, respectively. Total chlorophyll content was measured by UV-Vis spectrophotometer and calculated as n mol/ml (Mane et al. 2010).

$$\text{Chl a} = (14.21 \times \text{OD}_{663} - 3.01 \times \text{OD}_{645}),$$

$$\text{Chl b} = (25.23 \times \text{OD}_{645} - 5.16 \times \text{OD}_{663}), \text{ and}$$

$$\text{Carotenoids} = \{1000 \times \text{OD}_{470} - (3.27 \times \text{Chl a} - 1.04 \times \text{Chl b})/5\}.$$

The obtained values were divided by leaf fresh weight to obtain values in n mol/ml/mg of leaf fresh weight.

### 4 Statistical analysis

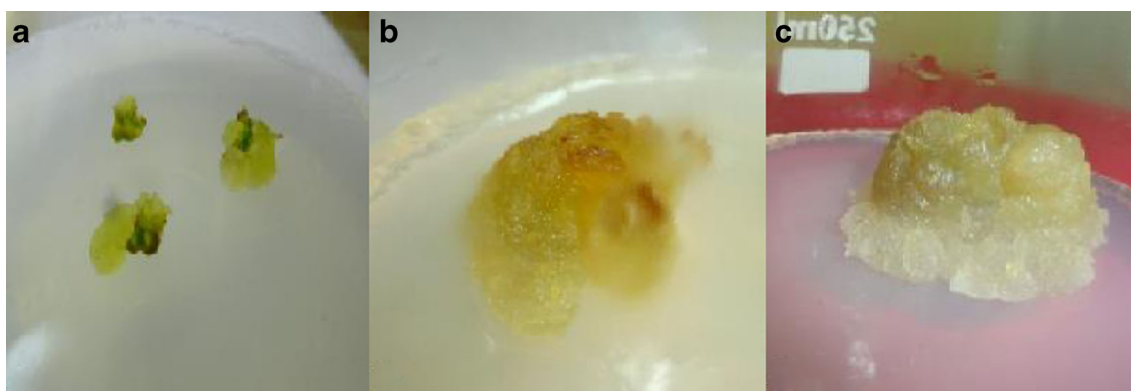
Morphological parameters such as number of shoot, shoot length, root length, fresh weight were examined using analysis of variance (ANOVA). Statistical analysis was carried out using SPSS software version 16.

### 5 Results and discussion

In the present study, we have studied the effects of *P. indica* filtrate on overall biomass productivity in *A. annua*. The basic goal of our study was to monitor the effect of *P. indica* on artemisinin production. The developmental stage of *Artemisia annua* at which maximum artemisinin concentration could be found is an important aspect. Majority of the investigators have reported the highest artemisinin content at the presence of pre-flowering stage (Liersh et al. 1986; Jha et al. 2010; Alam and Abdin 2011). Therefore, we have initiated the process by selecting node and leaf explants from the preflowering stage. Further, we established an efficient protocol which showed that the rate of successful regeneration was 80 % from the explants used.

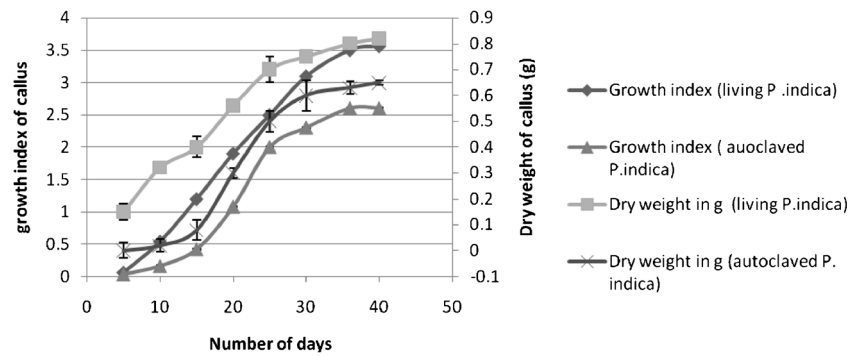
#### 5.1 Shoot initiation from nodal explants

Nodal explants inoculated onto MS+ BAP (3 mg/l) showed shoot growth after 5 days, and other cytokines showed initiation when it is used in combination (Fig. 1), while at 0.5 mg/l no shoot initiation was observed. Ram et al. 1997 also did not observe any shoot initiation at this concentration. Multiple



**Fig. 2** Leaf explants of *A. annua* showing callus culture. **a** Initiation of light green friable callus from leaf explants after 18 days of culture in MS medium with 2,4D. **b** Autoclaved *P. indica* treated callus turned brownish after 30 days of culture. **c** Callus treated with *P. indica* after 30 days of culture

**Fig. 3** Growth index and dry weight of *A. annua* callus culture treated with autoclaved *P. indica* and living *P. indica*. The values represent the mean of 5 independent measurement  $\pm$ S.E; all data are found to be significant at  $p < 0.05$



shoots development occurred at BAP (3 mg/l) within 30 days from a single node (Table 1). A total of 60 microshoots were obtained after 60 days of inoculation. Induction shoot with BAP (1.5 mg/l) was also reported in *A. Annua* (Dangash et al. 2015). Our improved protocol for direct shoot regeneration of *A. annua* using node explants on MS medium supplemented with BAP resulted in a rapid and high number of shoots. This regeneration system might be a useful method for high regeneration efficiency.

## 5.2 Root initiation from shoots

No root induction was observed in full MS at different concentration of IBA (1–4 mg/l). Dangash et al. (2015) also did not observe root induction at 1 and 2 mg/l IBA in full MS. Full MS supplemented with IBA (2–4 mg/l) showed callus formation instead at the base of microshoot. The root induction from the microshoots was observed in  $\frac{1}{2}$  MS + IBA (3 mg/l) within 8 days of inoculation. Initially, 2–3 roots were observed, but when transferred to the same medium a maximum of 18 roots were observed after 60 days. Dangash et al. (2015) observed root induction at 0.5 mg/l IBA at both full and  $\frac{1}{2}$  MS. They have reported that 0.5 mg/l NAA was the best concentration at both full and  $\frac{1}{2}$  MS for root initiation. They obtained 2–3 roots after 30 days of inoculation. But they have not studied the effect of either IBA or NAA beyond 2 mg/l. Our study

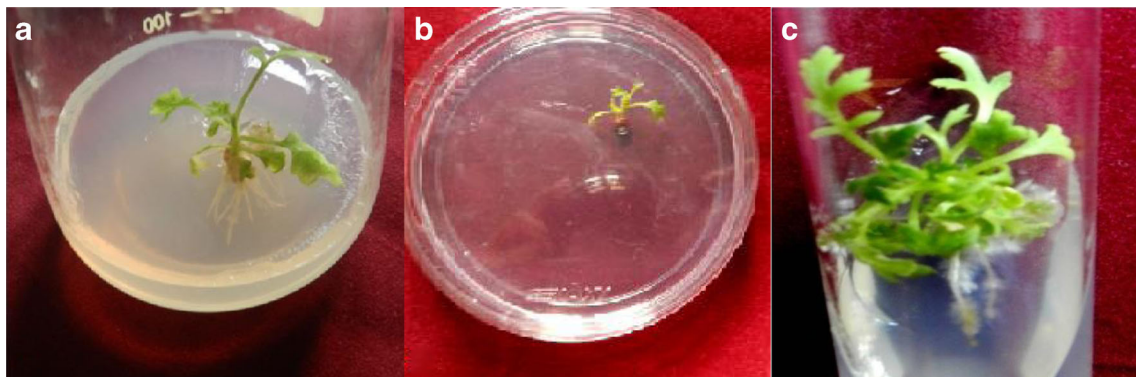
revealed that  $\frac{1}{2}$  MS medium containing higher concentration of IBA is effective in root initiation.

## 5.3 Establishment of callus culture and determination of callus growth index

The callus induction from leaf explants was observed in MS medium supplemented with 3 mg/l 2,4-D (Fig. 2). Light green, friable callus was observed after 18 days of inoculation. Similar texture of the callus was also reported by Nin et al. (1996).

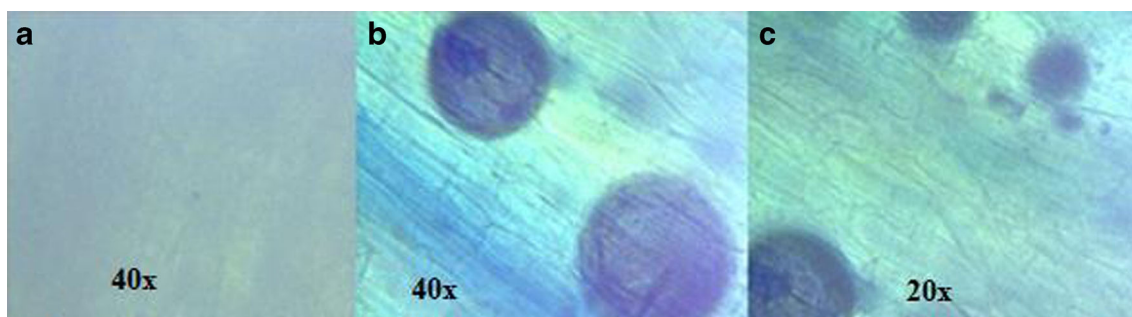
We studied the growth index of *Artemisia annua* callus treated with *P. indica* culture filtrate and autoclaved *P. indica* culture filtrate. There is no earlier report on callus growth in *A. annua* using *P. indica* culture filtrate. In this study, we observed that *P. indica* filtrate could not initiate callus, from the leaf explants, but it could effectively increase the growth of biomass compared to non-inoculated ones. Thus, the effect of *P. indica* culture filtrate is growth regulator dependent.

Callus inoculated with *P. indica* culture filtrate showed sigmoid shape curve. The dry weight and growth index of the callus increased gradually up to 35th day of culture. Further there was a steady growth, with a slight decrease in dry weight on 15th day and further increase in dry weight till reaches 40th day. Light green friable callus turned brownish colour after 40 days of culture. The callus inoculated with



**Fig. 4** Role of *P. indica* filtrate on *A. annua* root initiation and elongation. **a** root elongation takes place when microshoots were inoculated with *P. indica* filtrate after initiation of roots in  $\frac{1}{2}$  MS + IBA

(3 mg/l). **b** No root initiation was observed when *P. indica* filtrate was added before rooting in  $\frac{1}{2}$  MS + IBA (3 mg/l). **c** Control; Root initiation takes place when microshoots were inoculated in  $\frac{1}{2}$  MS + IBA (3 mg/l)



**Fig. 5** *P. indica* colonization on roots of *A. annua*. **a** autoclaved *P. indica* showed no chlamydospores in root cortex region. **b, c** *P. indica* colonization in treated plants of *Artemisia annua*

autoclaved *P. indica* also showed a sigmoid shape curve similar to the control. The fresh weight of the callus increased up to 25th day of culture; after that there is a decrease in growth. The callus turned brownish after 30th day of culture (Fig. 3). The dry weight of callus of control and autoclaved *P. indica* culture filtrate showed a decrease in dry weight after 30th day (Fig. 3). Thus we observed that *P. indica* culture filtrate has great impact on callus growth in *A. annua*.

#### 5.4 Effect of *P. indica* culture filtrate on root initiation and elongation

Attempts were made to achieve simultaneous root initiation and elongation with *P. indica* culture filtrate from in vitro derived shoots of *A. annua*. No root formation was observed when shoots were inoculated onto  $\frac{1}{2}$  MS medium supplemented with 3 mg/l IBA and *P. indica* filtrate (Fig. 4). This clearly shows that *P. indica* has a negative effect on root initiation. Similar result on the negative effects of the fungus for root initiation was also reported by Vyas et al. (2008). Similarly, micropropagated plums showed inhibition of rooting (Fortuna et al. 1998) using AM fungus and this root inhibition might be caused by immobilization of nutrients by AM fungus or competition for available nutrients, such similar behaviour might have caused suppressed root growth (Hetrick et al. 1988). Due to negative effect of the fungus for root initiation the shoots were allowed to root on  $\frac{1}{2}$  MS + IBA (3 mg/l) and then treated with *P. indica* filtrate. Root elongation in control, autoclaved *P. indica* filtrate and live culture filtrate were found to be  $5.8 \pm 0.03$ ,  $5.8 \pm 0.04$  and  $11.5 \pm 0.02$  respectively after 15 days of inoculation. This clearly signifies the role of *P. indica* culture filtrate in elongation of root in *A. Annua*.

#### 5.5 Effect of *P. indica* co-cultivation during hardening

The survival percentage of in vitro raised plantlets of *A. annua* during hardening was recorded to be 70 % in both the control and autoclaved *P. indica* treated plantlets, while it was found to be 96 % for *P. indica* treated plantlets in green house condition. The survival percentage after three weeks of hardening and acclimatization in potting mix under nursery shed was

75 % for both control and autoclaved *P. indica* treated plantlets, while it was 90 % in case of *P. indica* treated plantlets. Our result is supported by the works reported by Vyas et al. (2008) in *Feronia limonia*. They showed that the survival percentage of *P. indica* inoculated plants was 98 % in green house condition and 92 % in field condition.

#### 5.6 *P. indica* colonization

Successful root colonization with *P. indica* in the form of chlamydospore was observed on the root cortical region of

**Table 2** Growth parameters of *A. annua* plants treated with autoclaved *P. indica* filtrate and *P. indica* filtrate. Root and shoot length (in cm) increased *P. indica* filtrate inoculated plantlets. Similarly, the dry weight and fresh weight (in gm) were found to be higher in *P. indica* filtrate inoculated plants. The values represent the means of 5 independent measurements  $\pm$ S.E; all data were found to be significant  $p < 0.05$

	Control	Autoclaved <i>P. indica</i> filtrate	<i>P. indica</i> filtrate
Shoot length(cm)			
Day 0	2 $\pm$ 0.1	2 $\pm$ 0.1	2 $\pm$ 0.1
Day 5	2.6 $\pm$ 0.23	2.8 $\pm$ 0.04	5.2 $\pm$ 0.67
Day 10	5.0 $\pm$ 0.11	4.9 $\pm$ 0.05	7.8 $\pm$ 0.91
Day 15	5.4 $\pm$ 0.34	5.3 $\pm$ 0.88	9.4 $\pm$ 0.54
Root length(cm)			
Day 0	2 $\pm$ 0.1	2 $\pm$ 0.1	2 $\pm$ 0.1
Day 5	3.5 $\pm$ 0.78	3.3 $\pm$ 0.44	6 $\pm$ 0.50
Day 10	4.0 $\pm$ 0.89	3.8 $\pm$ 0.98	7.4 $\pm$ 0.87
Day 15	6.5 $\pm$ 0.05	6.8 $\pm$ 0.76	12.5 $\pm$ 0.67
Fresh weight(g)			
Day 0	6.2 $\pm$ 0.56	6.2 $\pm$ 0.56	6.2 $\pm$ 0.56
Day 5	13 $\pm$ 0.76	13.2 $\pm$ 0.33	24 $\pm$ 0.32
Day 10	15 $\pm$ 0.98	15.1 $\pm$ 0.04	25 $\pm$ 0.57
Day 15	34 $\pm$ 0.54	33.8 $\pm$ 0.09	50 $\pm$ 1.10
Dry weight(g)			
Day 0	2 $\pm$ 0.56	2 $\pm$ 0.56	2 $\pm$ 0.56
Day 5	3.2 $\pm$ 0.93	3.2 $\pm$ 0.34	6.2 $\pm$ 0.23
Day 10	5.2 $\pm$ 0.76	5.3 $\pm$ 0.06	9.2 $\pm$ 0.89
Day 15	9.2 $\pm$ 0.34	9.4 $\pm$ 0.76	12.0 $\pm$ 0.05

**Fig. 6** Overall growth and development of in vitro cultured *Artemisia annua* plant. **a, c** after 15 days and 25 days of inoculation respectively without *P. indica*. **b, d** after 15 and 25 days of inoculation respectively after co-cultivation with *P. indica*



in vitro grown plants treated with *P. indica* but it was devoid of chlamydo-spore in plantlets treated with autoclaved *P. indica* and control (Fig. 5). *P. indica*, has been reported to colonize the roots of a wide variety of plant species and promotes their growth (Kumar et al. 2011; Varma et al. 1999).

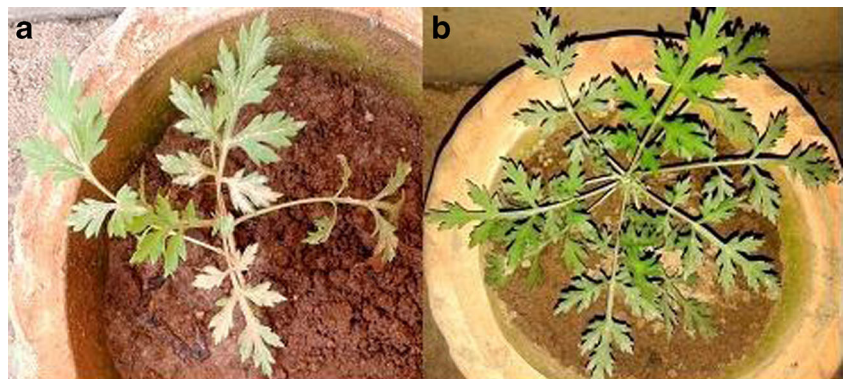
### 5.7 Growth parameters associated with *P. indica* filtrate

It is a well known fact that arbuscular and other mycorrhizal-like fungi play an important role in establishment, productivity and longevity of natural and man-made altered ecosystem (Prasad et al. 2012). *P. indica* mimics the typical arbuscular mycorrhiza (Varma et al. 1998) and it can be easily cultivated in axenic condition (Pham et al. 2004a, b) and it is associated with the roots of various plant species like mycorrhiza, promoting growth and sustainability (Varma et al. 1999; Varma et al. 2001; Deshmukh et al. 2006; Oelmüller et al. 2009; Bagde et al. 2010a, b, c).

A positive influence of the fungus, *P. indica* filtrate on overall growth and development of micropropagated plants of *Artemisia annua* was observed. Enhanced root length,

shoot length, dry weight and fresh weight were observed in *P. indica* colonized plants compared to control and autoclaved *P. indica* the autoclaved treated (Table 2). Sharma and Agrawal (2013) reported marked enhancement of artemisinin content and biomass productivity in *Artemisia annua* shoots by co-cultivating with *Piriformospora indica*. In the present study, we report for the first time that *P. indica* filtrate can be used to increase the biomass productivity in *A. annua*. There was an increase in stem size, number and colour of leaves, root length and root thickness (Fig. 6). However, no such enhanced growth was observed in autoclaved *P. indica*. Roots of *P. indica* inoculated plants were hard, thick and brownish compared to roots of plants treated with autoclaved *P. indica*. Morphological change in leaf size, leaf colour, stem thickness were observed within 5 days of co-cultivation. It is thus clear that *P. indica* treated plants showed better growth compared to non-inoculated or control plantlets (Fig. 7). Similar reports were observed for *Spilanthes calva* and *Withania somnifera* (Rai et al. 2001), *Bacopa monnieri* and tobacco. It has been proposed that *P. indica* improves plant growth by producing phytohormones, especially auxin

**Fig. 7** *A. annua* Plants showing biomass production. **a** Plants treated with autoclaved *P. indica* showed lower biomass compared to **b** plants treated with *P. indica* transferred after 3 weeks from green house to field condition



**Table 3** Photosynthetic pigment content of non inoculated and *P. indica* inoculated *Artemisia annua* plants. The values represent the means of 5 independent measurements  $\pm$ S.E; all data are found to be significant at  $p < 0.05$

	Control	Autoclaved <i>P. indica</i>	<i>P. indica</i>
<b>Chlorophyll a</b>			
Day 5	0.35 $\pm$ 0.01	0.35 $\pm$ 0.01	0.57 $\pm$ 0.01
Day 10	0.44 $\pm$ 0.02	0.46 $\pm$ 0.02	0.76 $\pm$ 0.05
Day 15	0.49 $\pm$ 0.03	0.49 $\pm$ 0.02	0.86 $\pm$ 0.04
<b>Chlorophyll b</b>			
Day 5	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01	0.20 $\pm$ 0.02
Day 10	0.14 $\pm$ 0.01	0.16 $\pm$ 0.01	0.25 $\pm$ 0.02
Day 15	0.19 $\pm$ 0.01	0.19 $\pm$ 0.01	0.29 $\pm$ 0.02
<b>Carotenoids</b>			
Day 5	34.5 $\pm$ 0.59	34.5 $\pm$ 0.59	47 $\pm$ 0.69
Day 10	36.0 $\pm$ 0.12	36.2 $\pm$ 0.04	52.0 $\pm$ 1.11
Day 15	37.7 $\pm$ 1.7	37.7 $\pm$ 1.7	58.0 $\pm$ 0.22

(Vadassery et al. 2008; Lee et al. 2011) which promotes branching of roots. Besides auxins, additional phytohormones such as cytokinins (Vadassery et al. 2008), gibberellins, abscisic acid and brassinosteroids (Schafer et al. 2009) have also been reported to be synthesized or manipulated by the root endophytes. In an earlier study, Lee et al. (2011) showed that *P. indica* induced growth promotion in Chinese cabbage and caused alteration in root morphology, such as adventitious root formation and root branching, which are controlled by auxin.

### 5.8 Photosynthetic pigment analysis

The interaction of the endophytic fungus with plant roots is accompanied by an enormous requisition of nitrogen from the environment (Wang et al. 2012). Nitrogen addition could enhance the photosynthetic capability of plant through increasing leaf chlorophyll content and the activity of rubisco, which is an important enzyme in photosynthesis. So, we measured the chlorophyll and carotenoid contents of autoclaved and non-autoclaved *P. indica* filtrate treated *A. annua* plants under in vitro condition. Chlorophyll a, chlorophyll b and carotenoid content were found highest in *P. indica* associated plants compared to the control and autoclaved *P. indica* treated plants (Table 3). Increased chlorophyll and carotenoid content due to presence of *P. indica* was previously reported in rice seedling by Jogawat et al. (2013).

## 6 Conclusion

The protocol developed for co-culturing with *P. indica* culture filtrate was found to increase the biomass productivity in

*A. annua* plant. However, further works need to be carried to determine the impact of this fungus on artemisinin content. Further, analysing the mechanism involved in biomass enhancement by this culture filtrate at molecular level may open up various new applications in the field of biotechnology, especially, herbal drug industry.

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