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Synergism study of *Bacopa monnieri* and *Piriformospora indica* and its impact on Biomass and metabolite

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Abstract

Background A symbiotic connection between *Piriformospora indica* and *Bacopa monnieri* (L.) Wettest, obtained through co-cultivation synergism, was found to improve growth, biomass production, and bacoside content in the plants. Brahmi (*B. monnieri* L.), a well-known Indian plant prized for its memory-boosting properties, has a lengthy history and a premium price tag. Because of its remarkable ability to colonize a wide variety of plant species, the axenically cultivable mycorrhiza-like endophytic fungus *P. indica* has gained a lot of interest recently.

Methods In the current study, fungal spores from recently revived cultures were added to jam bottles next to rooted Brahmi plants for in vitro co-cultivation. The control plants were left without fungal discs. Pre-rooted micro-propagated Brahmi plants were treated with agar discs containing actively growing hyphae. For a period of 3 months, both trials were conducted with a fully randomized setup. Microscopy of the treated and control plant roots verified co-cultivation.

Results Microscopic examination of the roots of co-cultivated plants reveals a high degree of colonization with host plants. These endophytic fungal structures include intracellular chlamydospores, and arbuscules, an intercellular and intracellular hyphae network, and a mycelial network on the root surface. In both in vitro and in vivo co-cultivation studies, the plant extended the host plant's lifespan in 3 months by displaying continuous regeneration; in contrast, the control plant displayed signs of senescence. With biomass exceeding the control by 1.18 times in vivo and 1.28 times in vitro. In vitro, co-cultivation circumstances also led to an increase in the rate of utilization of nutritional medium. In comparison to the control, the amount of bacoside increases to 100% in vivo after a month of co-cultivation and 33% in vitro after 3 months.

Conclusions In the present investigation, in vivo co-cultivation showed a favorable interaction effect on biomass production as well as bacoside content, which can satisfy the raw material demands of Brahmi plants in pharmaceutical industries.

Keywords *Bacopa monnieri*, *Piriformospora indica*, Co-cultivation, Symbiotic interaction, Synergism, Bacoside

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Background

Bacopa monnieri (L.) Wettest, a small creeping herb, commonly known as brahmi, belongs to the family Plantaginaceae (Sanyal et al. 2022). Brahmi is native to India and Bangladesh comprising 146 species of aquatic herbs distributed in different parts of the world, such as Nepal, India, Sri Lanka, China, Taiwan, Vietnam, Florida, and other US Southern regions (Abdul Manap et al. 2019). It has been identified among the seven important medicinal plants recommended for immediate attention and included in the list of highly endangered medicinal plants of India by the NMPB and TIFAC New Delhi (Saran et al. 2022), *B. monnieri* is a valuable medicinal herb used to enhance intelligence, memory (Mondal et al. 2023), and cognition (Abbott-Imboden et al. 2023). It contains a prominent active ingredient bacoside, and Triterpenoid Saponins, namely bacoside A and bacoside B, of which the former is important (Naik et al. 2017 and Banerjee et al. 2021). It has acquired popularity due to its nootropic properties and has been researched in vitro and in vivo for its antiasthmatic, anti-inflammatory, anticancer, antioxidant, cardiovascular, and hepatoprotective effects (Saloni et al. 2022). It is the most adulterated plant species among the medicinal plants used in Ayurvedic formulations, due to its huge demand and short supply. Hence, there is a need to find alternative ways for the production of bacoside A content of *B. monnieri*. Co-cultivation of plants with symbiotic microbes can be used to obtain a good yield of secondary metabolites under in vitro conditions.

In the complicated ecology of soil, there are several intricate interactions between the organisms. These crucial interactions in the soil greatly influence plant health. Numerous microorganisms, including a variety of ubiquitous arbuscular mycorrhiza (AM) fungi, work together to improve both the fitness of the individuals and the plants with which they are linked (Manchanda et al. 2017). Mycorrhizal fungi produce arbuscules in the root cortical cells of plants and form symbiotic relationships with the plants in which they exchange nutrients and carbon. In exchange, the fungus receives a carbon supply for biosynthesis and energy metabolism that comes from plant photosynthesis (Manchanda et al. 2017). Arbuscular mycorrhiza is responsible for the bidirectional exchange of water and nutrients, especially phosphorus and nitrogen for photosynthetic carbon to the mycorrhiza (Shi et al. 2023). Spores of *P. indica*, belonging to Basidiomycota, were isolated from the rhizospheric soils of *Prosopis juliflora* and *Zizupus numalaria* from the Thar desert of Western India by Verma and coworkers in 1998, it is now documented as *Serendipita indica* (Venneman et al. 2017 and Hyde et al. 2019). *Piriformospora indica* has been able to establish interactions with a wide range of host plants (145 plants). It develops symbiotic relationships with the host plant (Varma et al. 2014). It improves growth and biomass production of host plant (Varma et al. 2012). *Piriformospora indica* also works as a biofertilizer, bio-protector, immuno-regulator for the plants, and a biological mediator for the hardening of tissue culture grown plants (Varma et al. 2014). In a field experiment, Li et al. (2023a) and Li et al. (2023b) demonstrated the ability of *P. indica* to boost the growth, yield, and disease resistance of wheat plants. Its biofertilizer action is substantiated by several researchers. Such as Sherameti et al. (2005) reported growth promotion in Arabidopsis and tobacco plants through co-cultivation effect of *P. indica* which stimulated NADH-dependent nitrate reductase activity in roots resulted into enhanced nitrate uptake as well as expression of gene nitrate reductase (Nia2). It also express starch-degrading enzyme glucan-water dikinase in roots and shoots of host plant. There has been an increase in symbiotic N_2 fixation and/or nitrogen uptake, which could reduce the nitrogen demand (Beltayef et al. 2023). Kaboosi et al. (2022) reported *P. indica*, a plant growth-promoting biofertilizer, improves the blooming, yield, and physiological features of tomato *(Lycopersicon esculentum)* when employed as inoculants. Kaboosi et al. (2023) evaluated the effects of *Serendipita indica* (Synonym *P. indica*) on the growth and yield of maize plants under drought stress, in conjunction with a commercial biofertilizer. Shi et al. (2023) reported that *P. indica* has a wide host range, it colonizes the roots of host plants, which allows plants to grow under extreme physical and nutrient conditions. It acts as a biofertilizer in nutrient-deficient soils and promotes plant growth, induces early flowering, enhanced seed production, and stimulation of active ingredients in plants. Khalid et al. (2019) elaborated on the role of ethylene in establishing symbiotic interaction between *P. indica* and Arabidopsis mediated through *etr1, ein2, and ein2/eil1* genes, which promote the growth of host plants. They also reported that the Phosphate transporter gene (*PiPT*) in *P. indica* is actively involved in phosphate transportation and accumulates phosphate in the roots of host plants. Thus, the symbiotic relationship of *P. indica* with plants improves the nutritional status by accumulating nutrients in plants.

Also, various scientific reports state that *P. indica* acts as a bio-protector against biotic and abiotic stresses including root and leaf pathogens and insect invaders. According to Cheng et al. (2020), *S. indica* (Synonym *P. indica*) showed resistance against *Fusarium oxysporum* in Banana. Solanki et al. (2023) reported that *P. indica* promotes plant growth and also functions as a biofertilizer, phytoremediation, metabolic activity regulator, herbicide, and bio-pesticide. According to Li et al. (2023a), *P. indica* increases resistance to *Fusarium pseudograminearum*,

which causes fusarium crown rot in wheat, via stimulating the phenylpropanoid pathway.

Prasad et al. (2013) demonstrated the successful co-cultivation of *P. indica* with *B. monnieri* L. under in vitro conditions for 3 months and predicted the possibilities of enhancement in the plant growth, root proliferation, biomass production, and bacoside content in treated plants. Considering the above points, there is a need to confirm the interactive effects of *P. indica* on biomass as well as secondary metabolite production in *B. monnieri*. Keeping in view that the present investigation was carried out to study the effect of co-cultivation of *P. indica* on in vitro regenerated Brahmi plants, on growth, biomass production, and bacoside content of plants under in vitro as well as in vivo conditions.

Methods

Plant material and culture revival of *Piriformospora indica*

Brahmi plantlets were micro-propagated on sterilized MS medium (pH 5.8) supplemented with BA (1.1 µM), IBA (0.30 µM), and sucrose (30 g/L). These cultures were incubated for 4 weeks at 25 ± 2 °C with photoperiod of 16/8 h light and dark (Kharde et al. 2017). In vitro, regenerated micro shoots with 3–4 nodes, developed on hormone-free MS medium were transferred for root initiation on half-strength MS medium without a plant growth regulator (Sanyal et al. 2022). In vitro, rooted Brahmi plants were used for the in vitro as well as in vivo co-cultivation with symbiotic root endophyte *P. indica*. American type culture collection of *P. indica* (ATCC ®204458™) designated as strain-END1 (DSM11827) was procured from ATCC, USA through LGC Promochem India Pvt. Ltd. Bangalore, India and used for this experiment. *Piriformospora indica* culture was revived as per the procedure recommended by ATCC. Thawing of the frozen ampoule was done by placing it in a water bath at 25–30 °C for 5 min. Ampoules were just immersed in water up to the level that covered the frozen material without shaking. Immediately after thawing ampoules were wiped out with 70% ethanol and 50 µL culture from the ampoule was aseptically transferred to potato dextrose broth. The cultures were incubated at 30 °C and observed weekly for the growth of *P. indica* for 3 weeks. Revived colonies were subsequently subcultured on PDA by using the agar disc method; and incubated at 30 °C in the dark for 3 weeks. Further used for the co-cultivation study.

In vitro and in vivo co-cultivation

For in vitro co-cultivation, in vitro rooted Brahmi plants were treated with agar discs containing actively growing hyphae, and fungal spores from freshly revived cultures were placed in the jam bottles next to the rooted plants, while the control plants were kept as it is without fungal disc. Culture vessels were incubated in the growth room at 25 ± 2 °C and 16/8 h photoperiod for 3 months. The in vitro co-cultivation experiment was laid in a completely randomized design comprising two treatments, i.e., treated and control, with thirteen replications of each, while for the in vivo co-cultivation, the in vitro rooted plants were transplanted in pots by using sandwich layer method. The potting mixture has sterile soil layers at the top and bottom, while the middle layer contains a sterile sand-soil mixture and freshly subcultured *P. indica* culture containing viable spores as well as hyphae. For control, the in vitro rooted plants were transplanted into the pots containing only sterile soil. Experiment was laid in a completely randomized design with two treatments; treated and control and repeated three times each. These co-cultivated and control plants were maintained for 3 months and irrigated with sterile water without any fertilizer supplement.

After 3 months of incubation, treated as well as control plants were removed from the culture bottles. Roots were carefully cleaned with distilled water to remove traces of agar from the plants grown in vitro co-cultivation and similarly done with the pot-cultured plants grown in vivo co-cultivation to remove the adhered soil. Harvested plants were further observed for morphological variations, plant growth, and biomass production. Furthermore, aerial plant parts were used for the quantitative analysis of bacoside content, while the roots were used for the microscopic study to visualize the fuga–root interactions. Fungus-treated plants were compared with control plants in terms of morphological growth and secondary metabolite production (Prasad et al. 2013).

Microscopy

Mycelial growth on the co-cultivation media and the roots of the Brahmi plants were observed under a bright field light microscope (Labomed) by using lactophenol cotton blue and trypan blue (Merck) stains, respectively (Moreira et al. 2015). The root colonization and synergistic study of *P. indica* with Brahmi under in vitro and in vivo conditions were examined with the help of a light microscope. *Bacopa monnieri* roots were thoroughly washed and cut into small pieces approximately 1.0 cm in length. These root segments were treated with 10% w/v KOH (Merck) solution with constant heating for 10 min. Followed by neutralization with 1 N HCl and then washed with water. After that 0.05% trypan blue staining was done overnight and then these root segments were mounted in lactophenol on grease grease-free glass slide. These root segments were carefully covered with the coverslip to avoid air bubbles. Finally, these slides were observed under a light microscope (Prasad et al. 2013).

Bacoside analysis

Aerial plant parts of in vitro and in vivo co-cultivated crop were used for the quantitative analysis of bacoside content and compared with the control plant for statistical evaluation. Powdered samples were soaked with water for 24 h and squeezed out, followed by bacoside extraction with 95% (v/v) ethanol (AR Grade) (Paralel et al. 2010) and quantified by using a standard curve. A UV spectrophotometer was used to construct a standard curve at 278 nm using known concentrations of the standard bacoside (Sigma Aldrich) (Dowell et al. 2015). The results of the estimation of bacoside A content were further analyzed by using analysis of variance (ANOVA) at $P<0.05$, with the help of using WASP software developed by ICAR Coastal Agricultural Research Institute, Goa. Values were expressed as means of thirteen replicates in vitro co-cultivation as well as three replicates in vivo co-cultivation ± standard deviations (SD).

Results

Piriformospora indica culture

Piriformospora indica grown well on synthetic medium, colonies were developed and become visible within a week after inoculation and continued their further growth. *Piriformospora indica* cultured on PDA showed numerous spores under a light microscope after 2 weeks of inoculation. *Piriformospora indica* produced rhythmic rings when inoculated through agar disc on PDA (Fig. 1). It showed different growth and color patterns due to the pigmentation in the culture, where PDA culture showed irregular form of growth with undulated as well as lobate type of margins (Fig. 1a–d). To confirm the capability of co-cultivation with brahmi, *P. indica* was inoculated on MS basal medium and showed slimy-whitish growth on media.

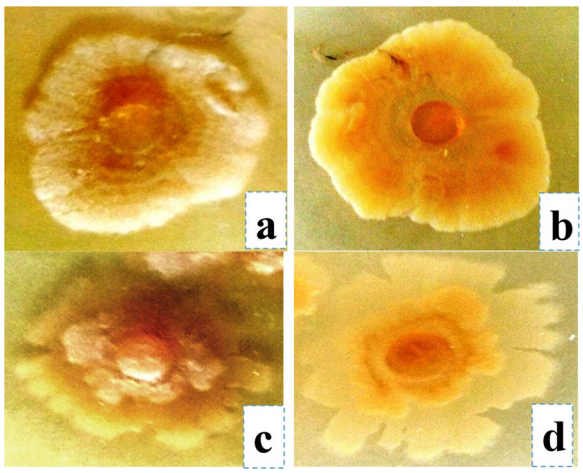

Fig. 1 Typical growth patterns of *P. indica* showing development of rhythmic rings in the culture, where PDA culture **a** and **b** were the ventral and dorsal view of culture on PDA after first subculture and **c** and **d** were the ventral and dorsal view of culture on PDA after second subculture

Morphology of co-cultivated plants

The present investigation showed the successful co-culture of *P. indica* and *B. monnieri* on MS media was investigated for 3 months. Under in vitro conditions, the co-cultivated plants demonstrated greater overall growth than the control plants (Table 1 and Figs. 2 and 3) as well as in vivo (Table 2 and Figs. 4, 5, and 6) conditions. After 3 months of inoculations and until complete media use, co-cultivated Brahmi plants were growing quickly and healthily, with green leaves and continual shoot multiplication (Figs. 2 and 4). Co-cultivated plants in both conditions show green foliage than the control, which might be due to the enhanced chlorophyll content of these plants. Thus, it indicates that there was an increase in longevity of the co-cultivated plants, while the control plants showed a natural phenomenon of senescence (Fig. 2b–d). Also, there were morphological changes, observed in vivo co-cultivated plants, such as leaf size and the stem diameter of these plants was more than the control (Fig. 4b), while the root color of freshly uprooted in vivo co-cultivated plant was light brown (Fig. 4c) and white control (Fig. 4d).

Utilization of culture media

Co-cultivated plants utilize almost complete nutrient media from the culture vessel, while the control plants fail to utilize the same (Fig. 2e). This showed that the fungus enhances the nutrient uptake capacity of the host plant and provides resistance against the stress conditions.

Table 1 Effect of in vitro co-cultivation of *B. monnieri* L. with *P. indica* growth, biomass production, and Bacoside A content

Growth parameter	In vitro co-cultivation	
	Control plant	*P. indica*-treated plant
No. of shoots	11 ± 1.63^{b}	20 ± 1.87^{a}
Number of root	44 ± 2.55^{b}	58 ± 3.34^{a}
No. of nodes	85 ± 5.87^{b}	103 ± 3.39^{a}
No. of leaves	175 ± 3.96^{b}	210 ± 5.10^{a}
Shoot length (cm)	5.59 ± 0.27^{b}	5.98 ± 0.10^{a}
Fresh wt (gm)	2.6 ± 0.15^{b}	7.1 ± 0.32^{a}
Dry wt (gm)	0.17 ± 0.03^{b}	0.67 ± 0.03^{a}
Bacoside A (mg/g DW)	3.6 ± 0.17^{b}	4.8 ± 0.17^{a}

Data analyzed by analysis of variance (ANOVA) at $P < 0.05$

Mean values within row followed by the different letters differ significantly at 5% level of probability

Values expressed are means of thirteen replicates ± SD

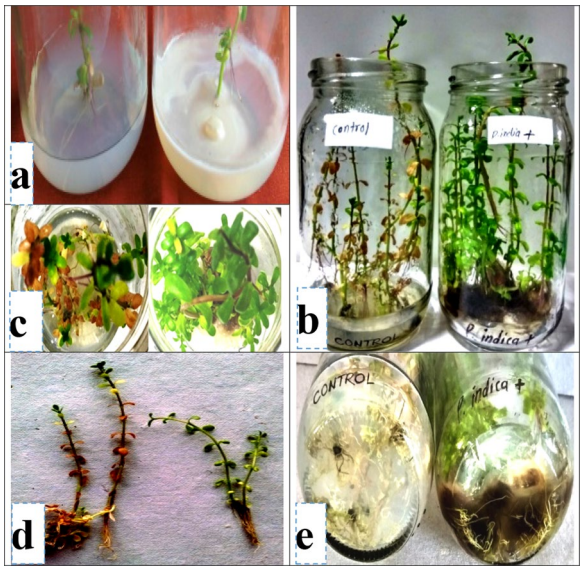

Fig. 2 Effect of co-cultivation of *P. indica* on growth of *B. monnieri* L. under in vitro conditions after 3 months on the growth and biomass production, where fig **a** treated plants shows *P. indica* growth on MS media in co-cultured culture vessel (right) and control plants (left), **b**–**d** treated plants (right) shows vigorous growth than control (left), **c** and **d** treated plants (right) shows regenerative growth with high photosynthetic pigmentation than control plants (left), which shows senescence, **e** treated plants (right) utilizes complete media, while control plants (left) fails to do so

Plant growth, biomass production, and bacoside content of co-cultivated plants

Overall growth of co-cultivated Brahmi plants with *P. indica* was increased than control under in vitro (Table 1 and Figs. 2 and 3) as well as in vivo (Table 2 and 4; Figs. 4, 5, and 6) conditions.

In vitro co-cultivation

In vitro, co-cultivation showed an increase in the number of average shoots, nodes, and leaves (that was 1.18, 1.21, and 1.20 fold, respectively) with continuous shoot proliferation over the control (Table 1 and Figs. 2 and 3). Also, there was an increase in average height (5.98 cm) than the control plants (5.59 cm). Co-cultivation enhanced biomass production in treated plants, such as mean fresh weight (7.1 g), and dry weight (0.67 g), while the control showed mean fresh weight (2.6 g), and dry weight (0.17 g). Bacoside content was also enhanced by the co-cultivation effect in Brahmi plants as compared to control plants, i.e., 4.8 mg/g in co-cultivated plants, while 3.6 mg/g in control plants.

In vivo co-cultivation

In vivo co-cultivation enhances the growth of the co-cultivated plants after 3 months as compared to the control plant (Table 2 and Figs. 5 and 6). Co-cultivated plants showed an increase in shoot proliferation monthly as 1.83, 1.61, and 2.16 fold increase in the first, second, and third months over control, with a maximum of 70 and 32 shoots in treated and control plants, respectively. Shoot length in the third month was 28.65 cm was enhanced by 1.56 fold over the control (18.31). Likewise, the number of nodes and leaves in the third month increased by 2.34 and 2.33 folds over the control, respectively. Root biomass was also increased in co-cultivated plants over the control (Fig. 7c–d). Thus, co-cultivation enhanced the growth of treated plants, which can be due to the

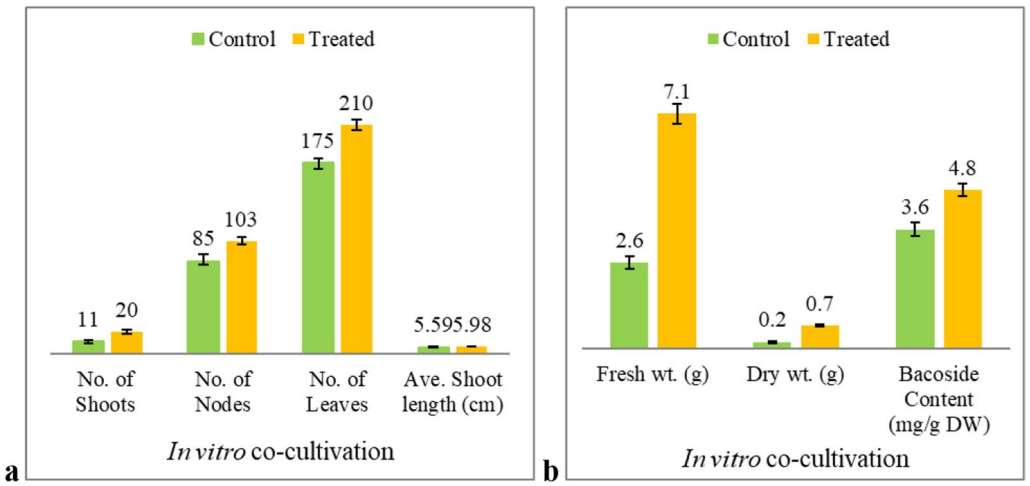

Fig. 3 Effect of in vitro co-cultivation of *B. monnieri* L. with *P. indica* for 3 months on **a** growth and **b** biomass production (Fresh and Dry wt.) and bacoside content. Bars indicated standard deviation

Table 2 Effect of in vivo co-cultivation of *B. monnieri* L. with *P. indica* on growth, biomass production, and Bacoside A content

Growth parameter	In vivo co-cultivation					
	1 month		2 months		3 months	
	Control	*P. indica* treated	Control	*P. indica* treated	Control	*P. indica* treated
No. of shoots	6 ± 1^{b}	11 ± 2^{a}	13 ± 2^{b}	21 ± 3^{a}	32 ± 3^{b}	70 ± 5^{a}
No. of roots	11 ± 2^{b}	17 ± 3^{a}	18 ± 2^{b}	31 ± 4^{a}	22 ± 4^{b}	49 ± 6^{a}
No. of node	26 ± 2^{b}	67 ± 6^{a}	116 ± 5^{b}	287 ± 3^{a}	211 ± 8^{b}	494 ± 6^{a}
No. of leaves	58 ± 4^{b}	134 ± 5^{a}	238 ± 6^{b}	580 ± 7^{a}	427 ± 10^{b}	998 ± 16^{a}
Shoot length (cm)	9.71 ± 0.06^{b}	15.2 ± 0.7^{a}	12.64 ± 0.08^{b}	23.12 ± 0.17^{a}	18.31 ± 0.14^{b}	28.65 ± 0.18^{a}
Fresh wt (gm)	2.2 ± 0.23^{b}	21.1 ± 0.90^{a}	7.51 ± 0.16^{b}	32.12 ± 1.62^{a}	16.33 ± 0.58^{b}	40.49 ± 0.29^{a}
Dry wt (gm)	0.17 ± 0.04^{b}	1.93 ± 0.08^{a}	0.58 ± 0.05^{b}	2.92 ± 0.08^{a}	1.25 ± 0.07^{b}	3.68 ± 0.04^{a}
Bacoside A (mg/g DW)	0.003 ± 0.001	0.006 ± 0.002	0.003 ± 0.001	0.0012 ± 0.001	0.01 ± 0.002^{a}	$0.003 \pm 0.001_{b}$

Data analyzed by analysis of variance (ANOVA) at $P < 0.05$

Mean values within row followed by the different letters for respective month differ significantly at 5% level of probability

Values expressed are means of three replicates ± SD

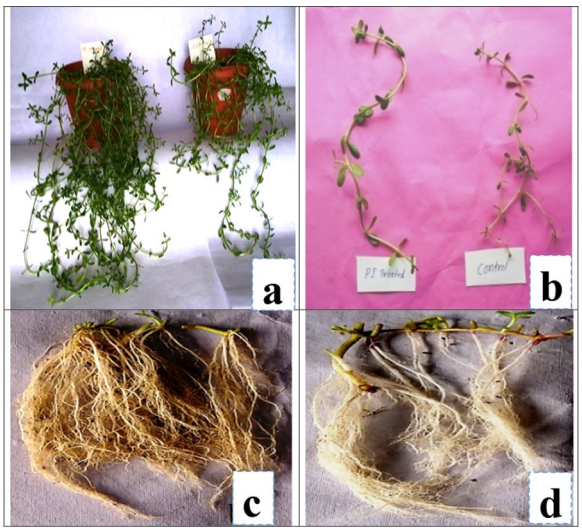

Fig. 4 Effect of co-cultivation of *P. indica* on growth of *B. monnieri* L. under in vivo conditions after 3 months on the growth and biomass production, where *P. indica* treated plants (left) and the control plants (right) fig. **a** and **b**, respectively, **a** Treated plants (left) shows increased growth than control, **b** treated plants (left) also increased size of leaf and stem than the control (right). While, vigorous root growth was observed in treated plants (**c**) than control (**d**)

interaction effect of the fungus. Biomass production of the aerial plant parts was enhanced in co-cultivated plants than in control; the fresh weight of aerial parts of co-cultivated plants was increased by 9.55 fold over control in the first month. However, the rate of increase in fresh weight of co-cultivated plants over the control was decreased periodically in the second month fresh weight of treated plants was increased by 4.28 fold, and in the third month that was 2.94 fold increase in weight as compared to the control. Similarly, dry weight increased at a slower rate, with 11.35, 5.03, and 2.94 fold increases in dry weight of aerial plant portions of co-cultivated plants over the control. Thus, the present investigation showed maximum enhancement in biomass production in co-cultivated plants was achieved in the early stage of the co-cultivation. Bacoside A content of in vivo co-cultivated plants was doubled (0.006 mg/g DW) in the first month of co-cultivation to control (0.003 mg/g DW). Surprisingly in the second month bacoside 'A' content of the control remained constant. However, when compared to the previous month, the treated plants showed a five-fold drop. Unpredictably, in the third month of co-cultivation bacoside 'A' content of co-cultivated plants showed again increase by 2.5 fold as compared to the second month, while the control plants showed more increase in bacoside content and produced maximum bacoside (0.01 mg/g DW) that was 3.33 fold as compared to the second month.

Microscopy of co-cultivated plant roots

Results of the microscopic study are depicted in Fig. 7. After 3 months of co-cultivation *P. indica* grown on MS medium in the culture bottle of co-cultivated plants. It was confirmed that a microscopic study showed the typical mycelium growth with sporulation at the tip of the hyphae. Chlamydospores were observed on the septate mycelial hyphae grown on MS media, typical pear-shaped, and globular chlamydospores (Fig. 7a–b). Numerous fungal spores were present in root hairs of co-cultivated plants, while they were absent in control plant roots, (Fig. 7c–d). Fungal colonization was observed on the root surface as well as in the intercellular and intracellular space in the cortex cells of the root as well as the

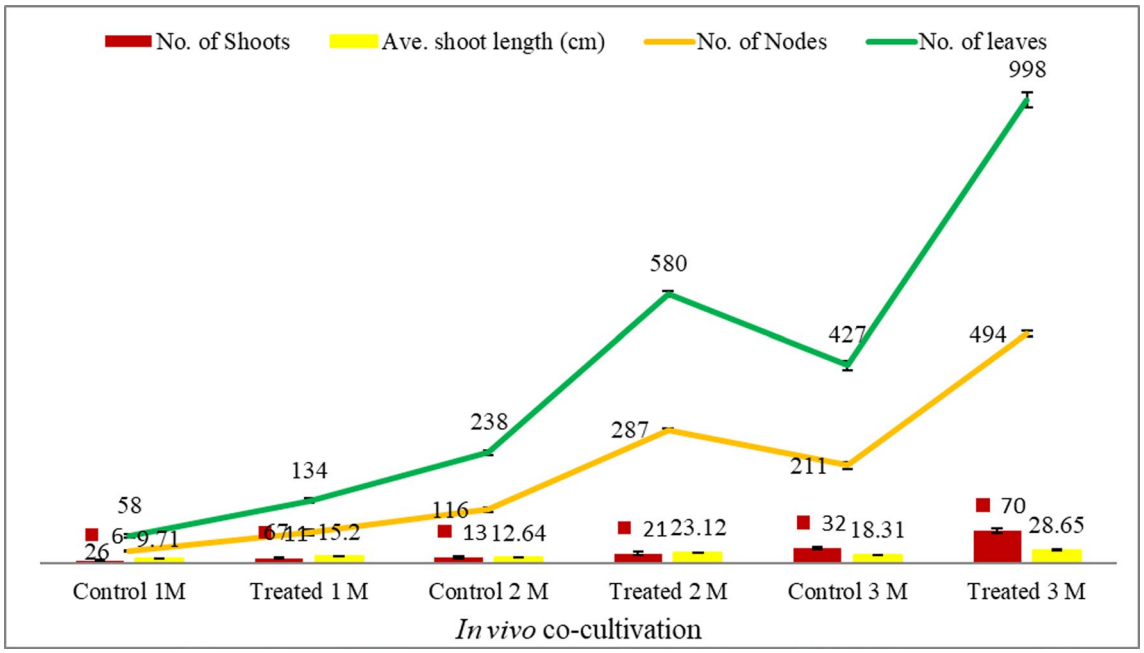

Fig. 5 Effect of in vivo co-cultivation of *B. monnieri* L. with *P. indica* for 3 months on growth of plant, where 1 M = 1 month, 2 M = 2 months, 3 M = 3 months. Bars indicated standard deviation

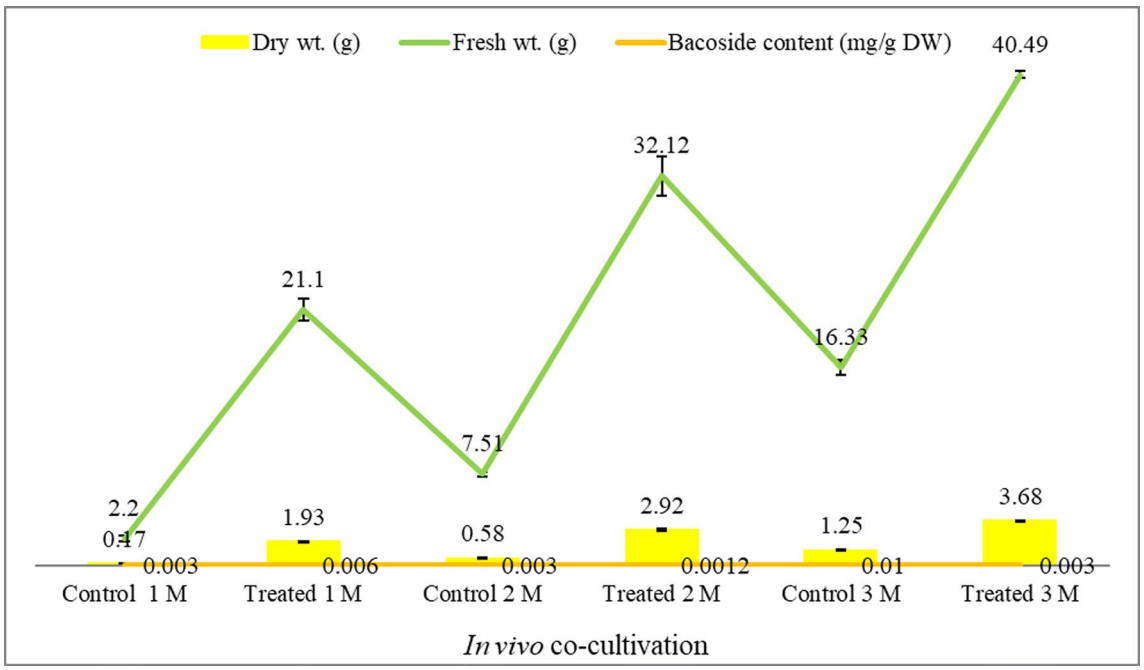

Fig. 6 Effect of in vivo co-cultivation of *B. monnieri* L. with *P. indica* for 3 months on biomass production (Fresh and Dry wt.), where 1 M = 1 month, 2 M = 2 months, 3 M = 3 months. Bars indicated standard deviation

root tip (Fig. 7e–f). Co-cultivated Brahmi roots showed fungal hyphae penetrated in the root cells (Fig. 7g–h). Root cells also showed several arbuscules inside the cells (Fig. 7i). However, a unique event of spore germination was observed in co-cultivated plant roots viz. typical spore, germinating spore with emerged hyphae, elongated independent hyphae (Fig. 7j).

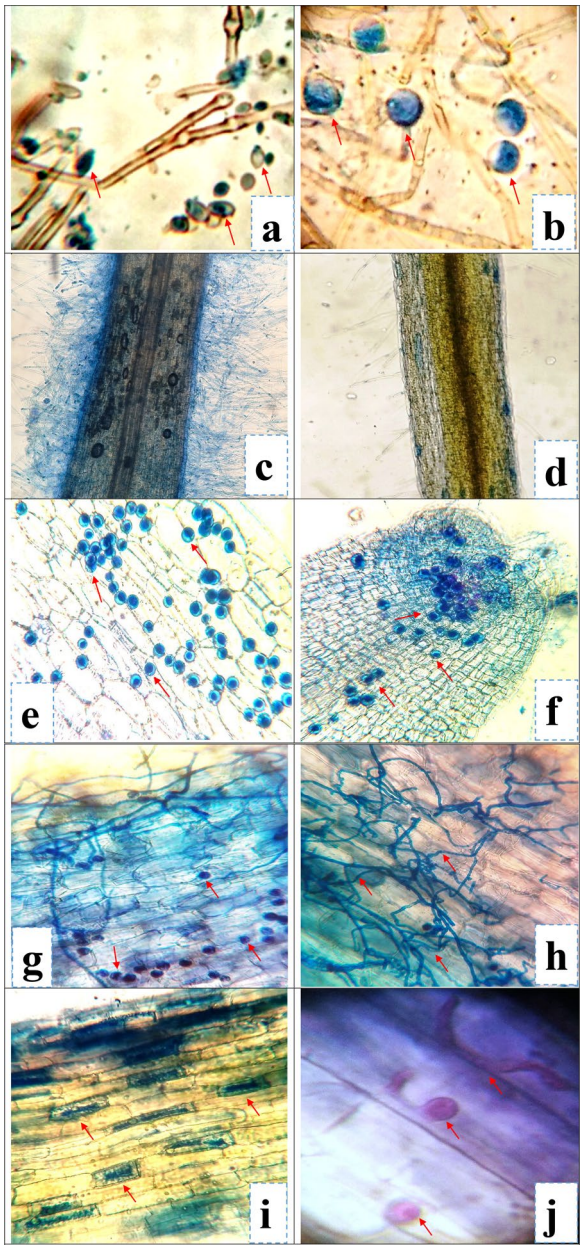

Fig. 7 Microscopic observations of roots of co-cultivated *B. monnieri* L. with *P. indica* 3 months, roots were observed under light microscope with magnification of 400× using with trypan blue stain. Septate hyphae with pear shaped (**a)** and globular chlamydospores (**b)** grows on culture media in co-cultivated plants under in vitro, **c** treated plant root, **d** control plant root, mature chlamydospores inside the roots (**e**) and root tip (**f**) of co-cultivated plants, intercellular hyphal network on root surface (**g**) as well as inside the root (**h**) of co-cultivated plants, intracellular arbuscules developed in co-cultivated plants roots. **j** typical fungal developmental stages were observed in adjacent cells in treated plant root, viz. chlamydospore, emerging hyphae from germinating spore and elongated hyphae

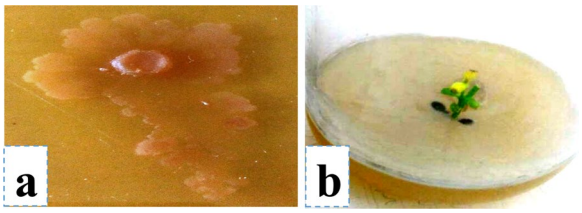

Fig. 8 Influence of long-term culture on growth and interaction ability of *P. indica,* **a** growth of *P. indica* cultured for more than 5 months after revival on PDA, **b** loss of interaction ability, co-cultivated *B. monnieri* L. plants with *P. indica*

Effect of long-term axenic culture on colonization efficiency of *P. indica*

In an independent experiment, *P. indica* cultures were maintained on PDA for more than 5 months. It showed varied growth patterns on PDA (Fig. 8a) and it failed to interact with *B. monnieri* plants when co-cultivated under in vitro conditions. The co-cultivation showed adverse effects on the host plant explant and resulted in the death of the co-cultivated plant within a couple of weeks after inoculation (Fig. 8b). Hence, there is a need for revival of the cultures before co-cultivation.

Discussion

Piriformospora indica culture

Piriformospora indica grown on PDA demonstrated noticeable growth within a week of inoculation and continued to proliferate. Microscopic examination revealed that numerous spores are formed in the culture after 2 weeks after inoculation. A similar type of growth of *P. indica* was observed by Hill and Kafer (2001) on the Kaefer medium. Rhythmic rings are the results of periodical growth patterns of mycelium, which stops for some time and produces several chlamydospores. Venneman et al. (2017) found that the mycelia development of *P. indica* was interrupted and restarted after 24–48 h, which produces chlamydospores and resulting in rhythmic growth.

Morphology of co-cultivated plants

The current study demonstrated successful co-cultivation of *P. indica* with *B. monnieri* L. on Murashige and Skoogs (MS) medium for 3 months. Under in vitro and in vivo circumstances, the co-cultivated plants demonstrated greater overall growth than the control plants. The first successful co-cultivation of *B. monnieri* with *P. indica* was performed by Prasad and coworkers, they reported enhanced growth and chlorophyll content of the treated plants (Prasad et al. 2013). Similarly, Ghorbani et al. (2021) revealed that the *P. indica* restored the chlorophyll

content of co-cultivated rice plants. Similarly, Jisha et al (2019) reported enhanced flowering in *Arabidopsis thaliana,* when interacting with *P. indica.*

Vadassery et al. (2008) revealed that the fungus produces relatively high levels of cytokinin in colonized roots of host plant as compared to the un-colonized roots of control plant, which has a vital function in controlling plant cell proliferation and differentiation, as well as plant growth and development, such as senescence delay. Kundu et al. (2021) revealed that the untargeted metabolite analysis using gas chromatography–mass spectrometry identified tomato (*Solanum lycopersicum*) compounds whose levels were altered during *P. indica*-mediated growth promotion. Metabolomic multivariate analysis indicated numerous primary metabolites with changed levels, with putrescine (Put) being the most significantly upregulated in roots during the interaction. Put increases root growth in tomatoes by increasing the amounts of auxin (indole-3-acetic acid) and gibberellin (GA4 and GA7). Kundu and Vadassery (2022) reviewed the numerous molecular pathways of *P. indica*-mediated growth promotion. Protein kinase-mediated pathways, enhanced food absorption, and polyamine-mediated growth phytohormone rise are a few examples.

According to Liu et al. (2023), root-associated endophytic fungi *P. indica* (Synonym *Serendipita indica*) modulates endogenous auxin and cytokinin levels in trifoliate orange (*Poncirus trifoliata*) seedlings. After 20 weeks of inoculation with these fungi, shoot and root biomass, root total length, taproot length, average diameter, surface area, volume, and the number of lateral roots all improved. It also considerably enhanced the concentrations of indoleacetic acid, indole butyric acid, trans-zeatin, dihydrozeatin, and isopentenyl adenine in the leaves and roots. It also increased plant growth and root architecture, which was linked to alterations in endogenous auxins and cytokinins. Correlation studies found that endogenous auxins and cytokinins were significantly positively related to both biomass and root morphological features (excluding root projected area). Inoculated plants showed higher expression levels of indoleacetic acid synthesis genes (*PtTAA1, PtTAR2, PtYUC3, PtYUC4, PtYUC6*, and *PtYUC8*) and indoleacetic acid transporter protein genes (*PtAUX1, PtLAX1, PtLAX2, PtLAX3, PtPIN1, PtPIN3, PtPIN4, PtABCB1,* and *PtABCB19*).

Utilization of culture media

Co-cultivated plants use almost all of the nutrient media from the culture vessel, demonstrating that the fungus improves the host plant's nutrient uptake ability and provides resilience to stress conditions, while the control plants do not. These results were in agreement with earlier reports, which supports the above statements of earlier researchers who reported that *P. indica* promotes nutrient uptake, and biomass production and provides the resistance in the host plants to survive under the stress conditions (Das et al. 2012 and Jisha et al. 2019). Also, Varma et al. (2012) reviewed that *P. indica* interacts with the host plant roots and obtains abundant quantities of nitrogen and phosphorous from the environment.

In vitro co-cultivation

In vitro, co-cultivation increased the number of average shoots, nodes, and leaves with continuous shoot proliferation above the control, and it also increased bacoside content in co-cultivated Brahmi plants. A similar type of result was observed by Prasad et al. (2013) in *B. monnieri* (L) Pennell, which showed an increase in the growth of *P. indica-treated* plants than the control after 3 months of co-cultivation. Also, Satheesan et al. (2012) reported that in vitro co-cultivation of *Centella Asiatica* with *P. indica* resulted in the enhancement in the root and shoot biomass as well as asiaticoside production.

In vivo co-cultivation

After 3 months, in vivo, co-cultivation increased the growth of the co-cultivated plants over the control plant. Lekshmi et al. (2022) reported that the *P. indica* colonized black pepper plants had considerably higher IAA levels. Indole-3-acetic acid, which promotes cell elongation and differentiation, must have boosted root development, which improved plant shoot rate.

Microscopy of co-cultivated plant roots

Microscopic examination of co-cultivated plant roots revealed characteristic endophytic fungal features such as a mycelial network on the root surface, intercellular and intracellular hyphae, intracellular chlamydospores, and arbuscules, indicating a high level of colonization with host plants. Moreira et al. (2015) detected a similar type of colonization by *P. indica* in pineapple plantlets 210 days after inoculation in a greenhouse with varying phosphorus levels in the soil. Li et al. (2023b) reported germinating chlamydospores on wheat root after 1 week of soaking treatments with chlamydospores suspension of *P. indica.*

Effect of long-term axenic culture on colonization efficiency of *P. indica*

Long-term axenic culture has negative effects and kills host plants since it cannot interact with them. Venneman et al. (2017) also showed that long-term storage cultures of *P. indica* lost vitality. As a result, there is a need for cultural revival before co-cultivation.

Conclusions

Microscopic observation confirms the interaction of inoculated fungus and host plants. These symbiotic interactions result in enhanced plant growth, biomass production, and increased secondary metabolite, i.e., bacoside content of the host plant. Overall, in vivo co-cultivation was more effective than in vitro cultivation revealing its potential for commercial applications. In vivo, co-cultivation enhances bacoside production in the early stage (first month) of co-cultivation, while in the case of in vitro co-cultivation, there is a need to study it for the early stages of co-cultivation rather than the 3 months.

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MK and SK are the major contributor for designed the study and supervised the experiment. AK carried out the experimental work and manuscript writing. RS reviewed the writing. All authors have read and approved the manuscript.

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Competing interests
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