https://doi.org/10.17221/119/2023-PPS

Piriformospora indica incumbers the incidence and growth of *Colletotrichum capsici* in colonized chilli plants by direct antagonism and activation of phenylpropanoid pathway

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Citation: Jojy E.T., Amrutha P., Johnson J.M., Sajeena A., Rafeekher M., Radhakrishnan N.V., Paul A. (2024): *Piriformospora indica* incumbers the incidence and growth of *Colletotrichum capsici* in colonized chilli plants by direct antagonism and activation of phenylpropanoid pathway. Plant Protect. Sci., 60: 354–370.

Abstract: Chilli anthracnose, incited by *Colletotrichum capsici*, is a major disease affecting the quality and quantity of chilli production. Farmers greatly depend on synthetic fungicides for the management of the disease. However, the extensive and non-judicious use of chemical fungicides resulted in the development of fungicide resistance in the pathogen and associated human and animal health risks. *Piriformospora indica*, a beneficial fungal root endophyte, has been employed as an efficient and safe biocontrol agent for managing bacterial, fungal and viral diseases and enhancing growth and yield. Hence, the present study was carried out to establish the protective role of *P. indica* against the chilli anthracnose incitant, *C. capsici*. The enzymes of phenylpropanoid pathway involved in this tripartite interaction were also studied. The study demonstrates that *P. indica* restricted *C. capsici* growth in dual culture with 57.22% mycelial inhibition on the 15th day after inoculation. *P. indica*-colonized chilli plants showed a delay in disease development, and significantly reduced the incidence and severity of chilli anthracnose disease compared to the control plants. Higher activities of defence-related enzymes viz. peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase, 4-coumaryl CoA ligase, cinnamyl alcohol dehydrogenase and total phenol in the *P. indica* – colonised plants revealed that the endophyte early resistance of plants against further pathogen invasions. The present study revealed *P. indica* to be an efficient biocontrol agent against chilli anthracnose. The results showed that *P. indica* reduced the infection of *C. capsici* by direct antagonism, activation of enzymes involved in plant defence and enhanced growth in chilli plants.

Keywords: *Piriformospora indica*; chilli; *Colletotrichum capsici*; dual culture; anthracnose; defence

Chilli anthracnose is a major disease limiting the production and productivity of the crop, leading to huge economic losses in the regions that cultivate chilli throughout the world. The dis-

Supported by the the Department of Science and Technology, Government of India.

https://doi.org/10.17221/119/2023-PPS

ease symptoms occur most commonly on leaves, stems and fruits. Among them, mature fruits are the most affected, reducing the marketable yield of chilli. Staggered pre- and post-harvest losses of 10–80% have been reported worldwide in field and storage conditions (Poonpolgul & Kumphai 2007). In India, the yield losses ranged 10–60% (Bansal & Grover 1969; Pooja & Simon 2019; Thilagam & Hemalatha 2019).

Anthracnose in chilli involves multifaceted aetiology as it is incited by different species of *Colletotrichum* fungi, viz. *C. gloeosporioides* Penz., *C. acutatum* Simmonds, *C. coccodes* (Wallr.) Hughes and *C. capsici* (Syd.) Butler and Bisby (Than et al. 2008a; Mongkolporn et al. 2010). *C. capsici* is India's most predominant incitant of chilli anthracnose and fruit rot (Chinthagunta & Zacharia 2018). Typical symptoms are observed as sunken or depressed, circular to angular, brown to black necrotic lesions on the infected fruit tissues, with concentric zonation of acervuli that produce orange or pink coloured conidial masses in addition to leaf spot, leaf blight, twig blight and dieback (Than et al. 2008b).

Anthracnose management mostly relies on the traditional practice of spraying chemicals such as dithiocarbamates and copper fungicides (Thind & Jhooty 1987; Rathore 2006). Continuous and generous use of chemical fungicides has led to fungicide resistance in pathogens and environmental, human, and animal health hazards. In the current scenario, biological control measures are emphasized, especially using beneficial endophytes, as these microbes persist in their host plants throughout the crop period. *Piriformospora indica*, a fungal root endophyte, has been recently employed for eco-friendly disease management in various crops. The fungus endophytically colonizes the roots of several crop plants, aiding in their growth promotion (Varma et al. 1999) and providing tolerance or resistance to various abiotic and biotic stresses. It also exhibits direct antagonistic effects by means of induced systemic resistance against various foliar and root pathogens (Baltruschat et al. 2008; Varma et al. 2012; Johnson et al. 2014; Gill et al. 2016; Ghorbani et al. 2021).

The endophyte was isolated from the roots of xerophytic shrubs (*Prosopis juliflora* and *Zizyphus nummularia*) found in Rajasthan. It is capable of growing on any complex media in the laboratory. The fungus is characterized by hyaline mycelium within the root cortex of colonized plants. Further, pear-shaped chlamydospores are produced inside the examined roots (Verma et al. 1998). *P. indica* is known for its plant growth-promoting effects by enhancing root and shoot lengths, flower and fruit production, and the final yield. In addition, the fungus also induces disease resistance and enhanced accumulation of secondary metabolites in host plants (Cheng et al. 2022).

We demonstrate that the root endophyte, *P. indica*, can potentially prime the defence of chilli plants against the foliar infection of anthracnose pathogen *C. capsici*, both *in vitro* and *in vivo*. The beneficial root endophyte enhances the activity of different enzymes, enabling its host plants to tolerate the pathogen attack and improving plant growth.

MATERIAL AND METHODS

Maintainence of *P. indica* and *C. capsici* cultures. *P. indica* (accession No. INBA3202001787) was cultured on sterilized potato dextrose agar (PDA) plates at pH 6.5, ambient temperature (25 ± 2 °C) and relative humidity (75 %), under 12 h light and 12 h dark conditions. For broth culture, 2–3 agar discs (5 mm) of fungal mycelia were transferred to 100 mL of sterilized potato dextrose broth (PDB) and maintained on a horizontal rotational shaker (50 rpm) till the complete mycelial growth (3 weeks).

C. capsici was isolated from chilli plants grown in experimental fields at the College of Agriculture, Vellayani. The pathogen was cultured on PDA medium (pH 6.5) and incubated at room temperature (25 ± 2 °C). Pathogenicity was proved by fulfilling Koch's postulates. Further, *C. capsici* was maintained by inoculating it into healthy chilli fruits and subsequent re-isolation from the infected fruit tissue, undertaken every six months to maintain its virulence.

Preparation of *C. capsici* spore suspension. Mycelial plugs (5 mm diameter) from *C. capsici* culture (two-week-old) were transferred to 100 mL of sterilized PDB (pH 6.5) and incubated at room temperature (27 ± 2 °C) till full mycelial growth and sporulation were obtained. Filtration of the medium was carried out using four layers of sterilized nylon membrane. Separated fungal mycelia and spores were carefully cleaned thrice in sterile double distilled water to eliminate any traces of the medium. Further, the fungus was slightly homogenized in water (50 mL) and passed through a sterile nylon

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membrane. The spores were subjected to a series of serial dilutions, and the concentration was adjusted to 10^6 spores per mL after counting using a haemocytometer. 50 mL of the prepared spore solution was supplemented with Tween-20 (1 drop) to allow uniform spore dispersion.

Dual culture assay of *P. indica* with *C. capsici*.Dual culture technique was used to evaluate the direct antagonism of *P. indica* against the pathogen *C. capsici.* Pure cultures of *P. indica* and *C. capsici* were maintained on PDA. 5 mm fungal discs were taken from 15-day-old *P. indica* and *C. capsici* cultures, placed opposite each other at one cm away from the peripheral edges of the 90 mm Petri plate. Control was maintained by placing a single species agar disc at one cm from the periphery of PDA plates. The inoculated plates were kept at room temperature (27 ± 2 °C), and mycelium growth of *P. indica* and *C. capsici* was recorded on the 1st, 2nd, 3rd, 5th, 7th and 10th day after inoculation.

The nature of antagonism following interaction between *P. indica* and *C. capsici* in a dual culture plate was also studied. Circular, sterilized cellophane sheets of 8 cm diameter were carefully placed over the PDA medium in Petri plates. Then, agar discs of *P. indica* and *C. capsici* were placed equally opposite, 1 cm from the edges of the same plate. After the inoculation, plates were undisturbed at room temperature (27 ± 2 °C) for a week. Antagonism involved in the *P. indica* – *C. capsici* interaction was assessed by observing the stained slides prepared using the cellophane sheet cut at the site where both *P. indica* and *C. capsici* met.

Co-cultivation of *P. indica* with chilli seedlings. Equal quantities of coir pith and dry powdered cow dung, mixed with 2 %-gram flour and 30–40% moisture, were sterilized for two consecutive days. Eighteen-day-old *P. indica* grown in PDB was added to the sterilized medium for the mass cultivation of *P. indica* as per the standardization procedure given by Jojy et al. (2020). *P. indica*-mass multiplied medium and normal medium devoid of *P. indica* were filled in separate clean portrays. Chilli seeds (var. Vellayani Athulya) were surface sterilized using 0.1 % sodium hypochlorite solution for 2 min, followed by three washings in sterile distilled water. The surface sterilized chilli seeds were planted in portrays and incubated in a green greenhouse for germination. At 15 and 30 days after co-cultivation, chilli seedlings were transplanted into small plastic containers and pots to evaluate *P. indica* priming against *C. capsici* infection.

Root colonization study. Ten *P. indica*-colonized chilli plants (15-day-old) were randomly selected, roots were cleaned, and placed in 10% KOH solution, heated at 90 °C for 3 min. The roots were later suspended for 1 min in 1N HCl, stained in lactophenol cotton blue solution (1 min) and photographed under the light microscope (Leica Microsystems DM300, Germany).

Assessment of *P. indica*-priming on control of *C. capsici*. The study involves two experiments. The first experiment was conducted under laboratory conditions. Fifteen-day-old chilli seedlings (var. Vellayani Athulya) propagated in the portrays were planted in clean, small plastic containers containing *P. indica*-multiplied medium and control medium (devoid of *P. indica*). Two weeks after transplanting (30-day-old seedlings), four treatments with twenty plants in each treatment were systematized for the study as follows: *P. indica*-colonized/primed plants alone (T_1), *C. capsici*-inoculated plants alone (T_2), *P. indica*-colonized/primed plants subsequently inoculated with *C. capsici* (T_3) and the control (T_4). Plants in treatments T_2 and T_3 were spray-inoculated with spore suspension (10^6 spores/mL) of *C. capsici* onto leaves. All the treated plants were covered with polythene bags to maintain 85% humidity.

In the second experiment, clean pots were filled with potting mixture comprising soil, cow dung and sand (1:1:1 w/w). One-month-old chilli seedlings from control and *P. indica*-primed plants were transplanted to the pots placed in the outdoor environment. Sixty-day-old chilli plants of the variety Vellayani Athulya were used for the experiment. Similar to the first experiment, there were four treatments with 20 plants each, viz., *P. indica*-colonized/primed plants alone (T_1), *C. capsici*-inoculated plants alone (T_2), *P. indica*-colonized/primed seedlings + *C. capsici* (T_3) and the control (T_4). Chilli plants in T_2 and T_3 combinations were spray-inoculated with pathogen spore suspension (10^6 spores/mL) onto leaves and enclosed within polythene bags to maintain humidity of 85%.

Inoculated plants in the experiments were carefully observed for the appearance of symptoms. Days taken for symptom development, lesion size (cm) and disease severity at 3, 5, 7, 10 and 15 days

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following inoculation were recorded. The root growths in the different treatments were also recorded. The standard disease chart given by Vishwakarma and Sitaramaiah (1986) was used to score anthracnose disease severity, where 0: healthy fruits, 1:up to 5%, 2: 5–25%, 3: 25–50%, and 4: 50–100% of fruit area affected. Percentage disease index (PDI) was calculated using the formula devised by Wheeler (1969):

$$PDI = \frac{\text{Sum of all the disease ratings}}{\text{Total number of plants observed} \times \text{Maximum disease grade}} \times 100$$

Estimation of phenols and defence-related enzymes. The experiment consisted of four treatments with five replications viz. *P. indica*-colonized/primed plants alone (T_1), *C. capsici*-inoculated plants alone (T_2), *P. indica*-colonized/primed seedlings + *C. capsici* (T_3) and the control (T_4). Leaf samples were taken from the treatments at 0, 12, 24 and 72 hours after pathogen inoculation (HAI).

Total phenols. Vidhyasekaran *et al*. (1992) outlined the method for phenol content estimation. 500 mg of the sample was boiled for 10 min in 10 mL of 80% ethanol and filtered to obtain the supernatant. This procedure was repeated four times to re-extract residue using ethanol (80%). All the extracts were pooled and air-dried to remove the remaining ethanol. The residue was dissolved in 2 mL of ethanol (80%) for total phenol estimation. The reaction mixture included 500 µL of Folin-Ciocalteau reagent, 100 µL of sample, and saturated sodium carbonate solution (1 mL). Sterile distilled water was supplemented in the mixture to make up the final volume of 3 mL. The water was boiled in the water bath for 5 min, followed by cooling under running tap water. The mixture was transferred to cuvettes, and using a spectrophotometer at 30 °C, absorbance was read at 725 nm. 100 mg of catechol dissolved in 100 mL of distilled water served as the standard. The working solution (10 µg, 20 µg, 30 µg, to 140 µg) was prepared by diluting the stock solution with distilled water in the proportion 1:10. The Standard graph plotted for catechol, where 'Y' is the concentration of total phenols in µg and 'X' is the optical density.

Peroxidase (PO) enzyme. PO enzymes (EC 1.11.1.7) are oxido-reductases widely distributed in plants, animals and microorganisms. They play key roles in growth, development and stress response in plants. POs are mainly involved in reactive oxygen species (ROS) metabolism, cell wall lignification and suberization. Thus aiding in the development of physical barriers by the synthesis of lignins, suberins, polysaccharides, and glycoproteins in cell walls. In addition, the production of ROS, phytoalexins, and pathogenesis-related (PR) proteins helps in plant defence response.

The activity of PO enzyme was assessed based on the procedure given by Srivastava (1987). According to this, the chilli leaf sample (100 mg) was pulverized in a chilled pestle and mortar using sodium phosphate buffer (5 mL) at pH 6.5, along with a pinch of polyvinyl pyrrolidone. This mixture was centrifugated at 6 000 rpm at 4 °C for 15 min. The sample cuvette included 0.05 M pyrogallol (3 mL) along with enzyme extract (50 µL), whereas pyrogallol alone was added to the reference cuvette. 1 % hydrogen peroxide (1 mL) was mixed to initiate the reaction. Reading of absorbance peak was taken at 420 nm using a spectrophotometer at 30 °C for 30 sec interval for 180 sec. PO enzyme activity is represented in mg/min × g fresh weight (FW).

Polyphenol oxidase (PPO) enzyme. PPO enzyme (EC 1.10.3.2) is popularly known as a fruit browning enzyme and well-distributed oxidative enzymes. These are induced in response to wound or pathogen attacks in plants. The enzyme catalyzes the conversion of polyphenols to o-quinones, thus reducing the acceptability of plant tissues and preventing the growth of pathogens.

PPO activity was evaluated by a procedure defined by Mayer et al. (1965). In a cooled pestle and mortar, 1 g of the leaf sample was crushed using sodium phosphate buffer (5 mL) at pH 6.5, and mixed with a small quantity of polyvinyl pyrrolidone. The supernatant was obtained by centrifuging the mixture at 6 000 rpm, at 4 °C for 15 min. Sodium phosphate buffer (1 mL) and enzyme extract (200 µL) were transferred to the sample cuvette, and the reaction was initiated by adding 0.01 M catechol (1 mL). The reference cuvette had a buffer alone. A spectrophotometer measured absorbance at 495 nm for 180 sec at 30 sec intervals at 30 °C. PPO activity is shown in terms of mg/min × g FW.

Phenylalanine ammonia-lyase (PAL) enzyme. PAL (EC 4.3.1.24) is a key enzyme in the phenylpropanoid pathway and is involved in the biosynthesis of flavonoids, lipids and other polyphenol compounds. Mainly, the enzyme catalyzes the conversion of L-phenylalanine to trans-cinnamic acid.

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Brueske (1980) detailed a protocol for the estimation of PAL activity. Refrigerated pestle and mortar were used to homogenize 500 mg of leaf sample using a 0.01 M sodium borate buffer (5 mL) prepared at pH 8.8. The homogenate was centrifuged at 4 000 rpm at 4 °C for 10 min. The resultant supernatant was saturated with 30 % ammonium sulphate and centrifuged for 10 min at 7 200 rpm. Saturation and centrifugation steps were repeated, and the resulting pellet was suspended overnight at 4 °C in 9 mL of sodium borate buffer. Sodium borate buffer (1 mL), enzyme extracts (1 mL) and phenylalanine (1 mL) were incubated at 32 °C for 60 min in a sample cuvette. The addition of trichloroacetic acid (0.5 mL) stopped the reaction, followed by 5 min incubation at 37 °C. 2 mg of cinnamic acid was mixed in 1 000 mL of distilled water and cinnamic acid concentrations in the range 0.1–10 µg/mL was used as standard. Absorbance reading was taken at 270 nm in a spectrophotometer at 30 °C, and PAL activity was assessed in terms of cinnamic acid formed in a minute per gram of sample (µg/g × min).

Cinnamyl alcohol dehydrogenase (CAD) enzyme. CAD (EC 1.1.1.195) is an important enzyme in the phenylpropanoid pathway. It is involved in the conversion of cinnamyl alcohols into cinnamaldehydes. This is the last step in monolignol biosynthesis for cell wall polymerization and lignification, aiding in plant defence.

CAD enzyme activity was estimated using the method described by Wyrambik and Grisebach (1975). Sodium phosphate buffer pH 7.3 (0.1 M) was added with 4 mM mercaptoethanol to pulverize 0.5 g of fresh leaf samples. Centrifugation of homogenate was done at 12 000 rpm for 10 min. One ml of 0.1 M cinnamyl alcohol, NADP (0.1 M), and Tris HCl buffer (0.1 M) at pH 9.3 were added to a cuvette. Later, the reaction mixture was mixed with enzyme extract (0.2 mL). Spectrophotometric reading of peak absorbance was measured at 400 nm at 40 °C. CAD activity was measured by the oxidation of the substrate, coniferyl alcohol, to form coniferaldehyde. The activity of CAD was determined by nanokatal unit (nkat) per gram on a fresh weight basis, using the molar extinction coefficient (€) of NADPH as 2.10×10^4/M × cm.

4-coumaryl CoA ligase (4-CL) enzyme. 4-CL enzyme (EC 6.2.1.12) is involved in phenylpropanoid pathway and helps in plant growth and against biotic and biotic stresses. This enzyme mainly contributes to the formation of coenzyme A esters, which in turn are involved in lignin biosynthesis.

The 4-CL enzyme was estimated according to Knobloch and Hahlbrock (1977). Initially, the extraction buffer was prepared using Tris HCl (100 mM), 2-mercaptoethanol (5 mM) and glycerol (5%). Chilli leaf samples (500 mg) were homogenized in an extraction buffer (5 mL) at pH 7.8. The resulting extract was centrifuged at 23 000 g for 30 min. The reaction mixture (0.2 mL) consisting of Tris HCl (100 mM) prepared at pH 7.8, p-coumaric acid (0.1 mM), $MgCl_2$ (5 mM), CoA (0.3 mM), ATP (0.5 mM) along with enzyme extract (10 µL) were dispensed in to sample cuvette. In contrast, the reference cuvette contained all the reagents except CoA. 4-CL activity regarding coumaroyl CoA formation was measured at 333 nm in a spectrophotometer at 30 °C, using the extinction coefficient as 2.1×10^4/Mol × cm (Stockigt & Zenk 1975).

Statistical analysis. The data was analyzed using ANOVA, and the means were compared using the Least Significant Difference (LSD) at a 5 % significance level.

RESULTS

***P. indica* restricts *C. capsici* in the dual plate by antibiosis.** The root endophyte, *P. indica* and foliar pathogen, *C. capsici* were co-cultivated on PDA medium, and their nature of antagonism was studied. In dual culture, *C. capsici* growth was inhibited by *P. indica* despite being normal in control. Mycelial growth of *C. capsici* was limited to 3.9 cm in the presence of *P. indica,* instigating maximum inhibition of 57% on the 15th day after incubation (Table 1).

Antagonistic interactions triggered the formation of inhibition or lysis zone followed by antibiosis at the point of interaction between *P. indica* and *C. capsici* (Figure 1A). On extended storage of these dual culture plates, *P. indica* surpassed the inhibition area into the pathogen colony, initiating mycelial thickening in *C. capsici* and its lysis in the advanced stages (Figure 1B).

The microscopic study also revealed inhibition in the release of pathogen conidia by *P. indica* (Figure 1B) compared with the production of falcate conidia by *C. capsici*, in the absence of

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Table 1. Mycelial growth inhibition of *Colletotrichum capsici* by *Piriformospora indica* in dual culture technique observed on 3rd, 5th, 7th, 10th and 15th day after incubation

Treatments	Mycelial growth (cm)					Nature of mycelial growth
	3rd day	5th day	7th day	10th day	15th day	
Piriformospora indica (dual culture)	4.4 ± 0.2	5.1 ± 0.1	5.5 ± 0.2	5.5 ± 0.2	5.5 ± 0.2	Normal
Colletotrichum capsici (dual culture)	3.1 ± 0.3 (9)	3.6 ± 0.2 (30)	3.8 ± 0.2 (38)	3.8 ± 0.2 (54)	3.8 ± 0.2 (57)	Suppressed
P. indica (control)	5.2 ± 0.1	5.8 ± 0.2	6.7 ± 0.2	7.6 ± 0.2	9.0 ± 0.0	Normal
C. capsici (control)	3.4 ± 0.1	5.1 ± 0.3	6.3 ± 0.4	8.4 ± 0.3	9.0 ± 0.0	Normal

The endophyte, *P. indica* and pathogen, *C. capsici* were co-inoculated (dual culture) or inoculated individually (control) on the opposite sides of PDA plates. The plates were incubated at room temperature, and observations were made at specific time intervals

Values in parentheses denote the percentage inhibition in pathogen mycelial growth due to *P. indica*. The data is based on four independent experiments with ten replications in each treatment per experiment, and errors represent SEs

P. indica (Figure 1C). In contrast, *C. capsici* did not affect the number and size of chlamydospores or morphology of *P. indica* mycelia (Figure 2). The result impelled us to assess the endophytic potential of *P. indica* in reducing chilli anthracnose incidence and severity.

***P. indica* reduced anthracnose and fruit rot incidence and severity in the primed chilli seedlings.** *P. indica*-colonized/primed chilli seedlings were evaluated against *C. capsici* under laboratory conditions. Chilli seeds (var. Vellayani Athulya) were sown in *P. indica* mass multiplied medium and control medium. After transferring chilli seedlings to containers and pots, anthracnose incidence and severity in the primed-chilli plants were assessed on the 3rd, 5th, 7th, 10th and 15th day after pathogen inoculation against control plants.

In the laboratory environment, initial water-soaked anthracnose lesions on leaves were produced on the third day in pathogen-inoculated *P. indica*-primed seedlings and on the second day (early) in pathogen-inoculated control. At seven days after inoculation (DAI), the lowest lesion size of 1.3 cm was recorded in leaves from *P. indica*-primed chilli seedlings, whereas the lesion size was 2.1 cm in the control plants. Further, *C. capsici*-inoculated leaves in the *P. indica* non-colonized plants showed complete rotting at 10 DAI. In comparison, the rotting of leaves was delayed to 15 DAI in the endophyte-colonized seedlings. Chilli plants colonized with *P. indica* or not in-

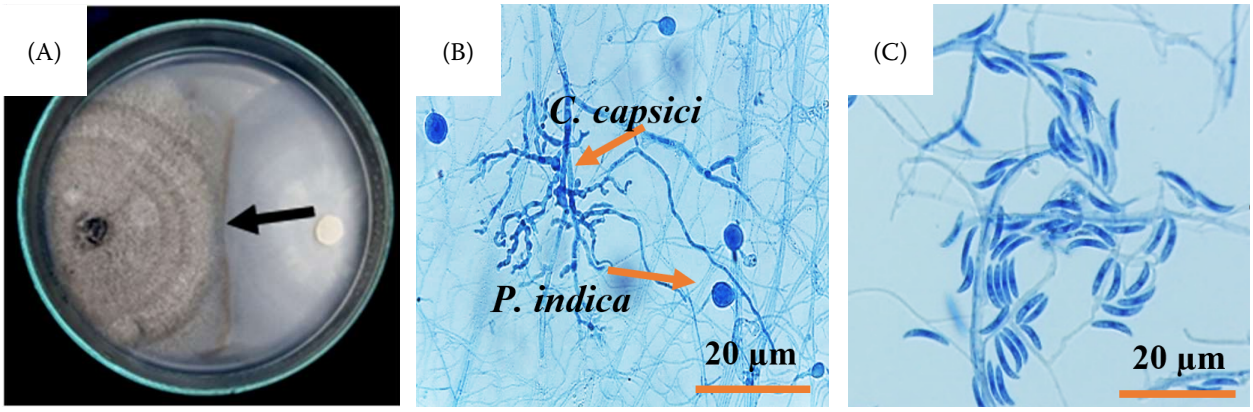

Figure 1. *Piriformospora indica* inhibits the growth of chilli anthracnose pathogen, *Colletotrichum capsici* in dual culture

(A) The picture shows the growth of *C. capsici* (brown) and *P. indica* (white) on same PDA plate about 13 days after incubation; arrow indicates inhibition zone followed by antibiosis at the interaction site between *P. indica* and *C. capsici*; (B) microphotograph from the stained co-cultures represents thickening and branching of *C. capsici* mycelium by *P. indica*; (C) the photograph shows spore production in *C. capsici*, without *P. indica*

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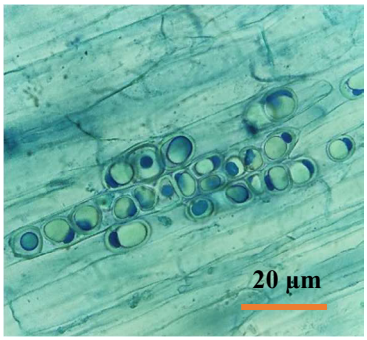

Figure 2. Pear-shaped chlamydospores of *Piriformospora indica* is observed within the primed roots of chilli seedlings
The picture shows the microscopic view of *P. indica* chlamydospores in the cortical cells of chilli root, stained with lactophenol blue after 15 days of colonization

oculated with any fungi did not exhibit any disease symptoms during the experimental period (Figures 3A–3D). Further, *P. indica*-primed plants recorded the lowest anthracnose disease severity of 30.63 % compared to control plants (77%) at 7 DAI (Figure 3E).

P. indica also enhanced the root and shoot biomass in the primed chilli seedlings. Primary roots produced numerous secondary as well as tertiary roots. Chilli plants colonized with *P. indica* had higher root length than control. In seedlings inoculated with *C. capsici*, secondary and tertiary roots and root length were significantly reduced (Figures 4A–4D). At 30 DAI, the highest root and shoot weights of 0.12 g and 0.17 g were recorded in *P. indica*-primed seedlings compared to 0.05 g and 0.11 g in control. Even after *C. capsici* inoculation, *P. indica*-colonized chilli seedlings exhibited significantly increased root and shoot biomass (0.09 g and 0.15 g) than control seedlings inoculated with the pathogen (0.03 g and 0.08 g) (Figures 4E–4F).

In the pot culture experiment (outdoor environment), the appearance of anthracnose symptoms on leaves was delayed by four days in *P. indica*-colonized plants, as opposed to two days in control. Similarly, on chilli fruits, fruit rot symptoms occurred only after six days in *P. indica*-primed plants compared to two days in control. After ten days of pathogen inoculation, leaves and fruits from *P. indica*-primed chilli plants produced a significantly lower anthracnose lesion size of 1.6 cm and 1.2 cm, respectively, compared to 2.9 cm and 3.6 cm in non-primed, *C. capsici*-inoculated chilli plants (Figures 5A–5D). *C. capsici*-inoculated control plants showed complete rotting of leaves and fruits on the 15th DAI. In contrast, the chilli plants exposed to the endophyte were still intact with considerably smaller lesions.

Similarly, *P. indica*-colonized chilli plants showed a considerable reduction in disease severity due to anthracnose compared to the non-colonized

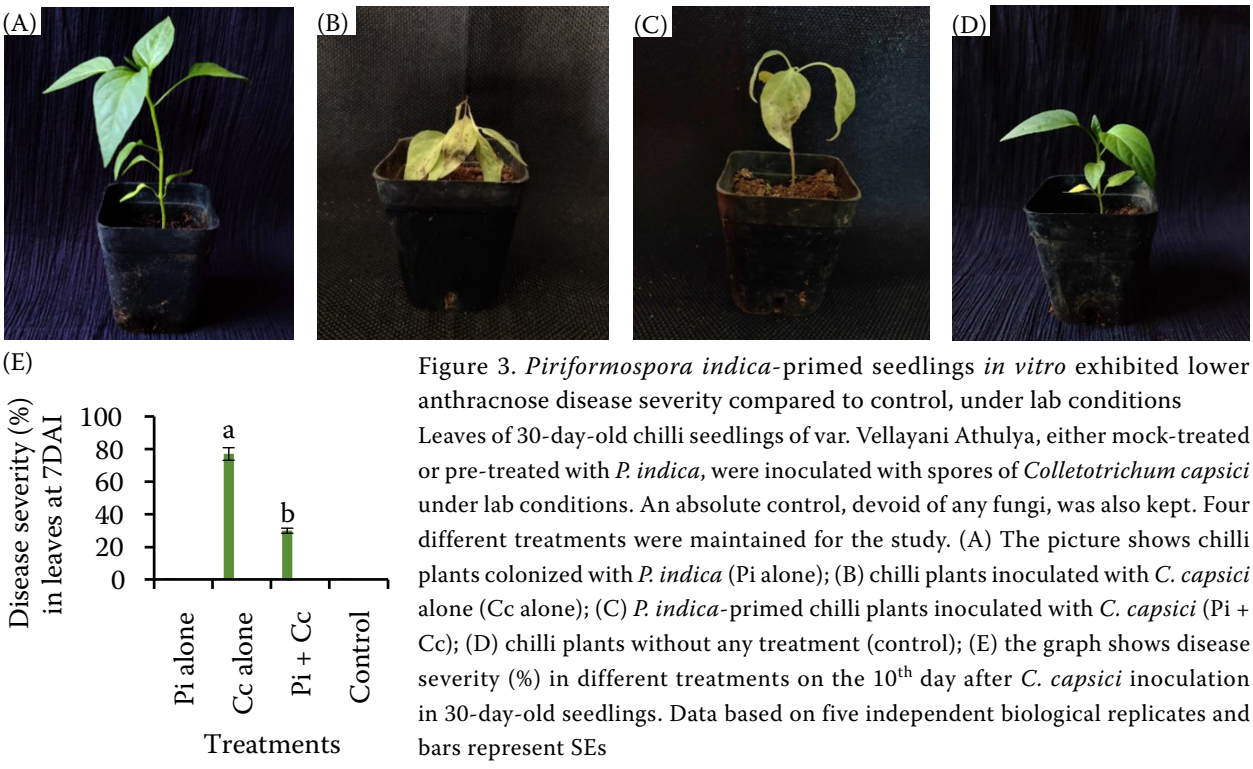

Figure 3. *Piriformospora indica*-primed seedlings *in vitro* exhibited lower anthracnose disease severity compared to control, under lab conditions
Leaves of 30-day-old chilli seedlings of var. Vellayani Athulya, either mock-treated or pre-treated with *P. indica*, were inoculated with spores of *Colletotrichum capsici* under lab conditions. An absolute control, devoid of any fungi, was also kept. Four different treatments were maintained for the study. (A) The picture shows chilli plants colonized with *P. indica* (Pi alone); (B) chilli plants inoculated with *C. capsici* alone (Cc alone); (C) *P. indica*-primed chilli plants inoculated with *C. capsici* (Pi + Cc); (D) chilli plants without any treatment (control); (E) the graph shows disease severity (%) in different treatments on the 10th day after *C. capsici* inoculation in 30-day-old seedlings. Data based on five independent biological replicates and bars represent SEs

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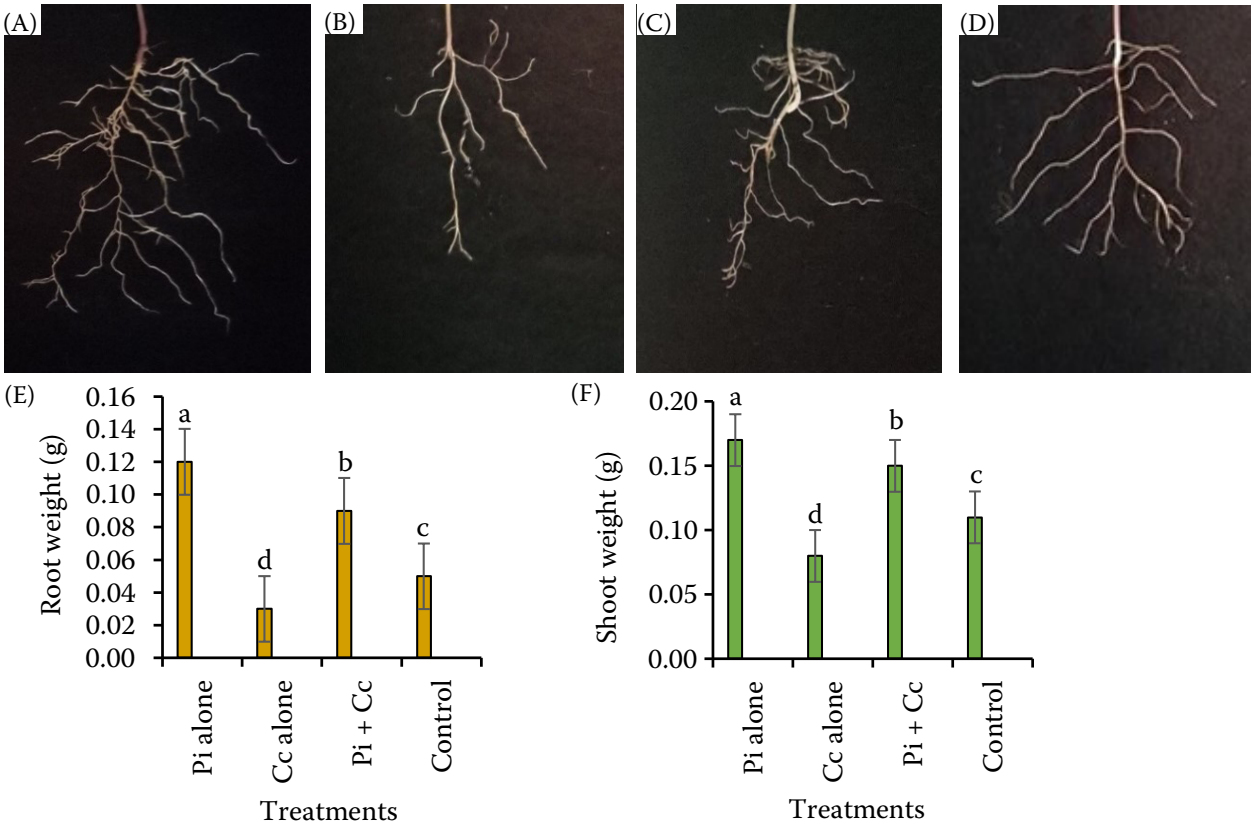

Figure 4. *Piriformospora indica* colonization enhances root and shoots growth in primed chilli seedlings (var. Vellayani Athulya)

The picture represents root biomass observed in four different treatments of the experiment. (A) *P. indica*-colonized chilli seedlings (Pi alone); (B) chilli plants inoculated with *Colletotrichum capsici* alone (Cc alone); (C) *P. indica*-primed chilli plants inoculated with *C. capsici* (Pi + Cc); (D) chilli plants without any treatment (control); the graph represents (E) fresh root weights and (F) fresh shoot weights of different treatments in 30-day-old chilli seedlings. Bars represent data from analysis of five independent biological replicates, and the significantly different values are represented by different letters above the bars

plants. *C. capsici*-inoculated leaves and fruits from *P. indica*-colonized plants exhibited slow advancement in percentage disease index (PDI), which reached 35% and 12%, respectively, compared to 75% and 68% in the control plants on 7th DAI. A lower % anthracnose disease severity of 62% was observed in *P. indica*-primed chilli leaves compared to the pathogen alone (94%) at 10 DAI. Meanwhile, fruits from *P. indica*-primed plants recorded the lowest disease severity at 28%, compared to control plants (92%) (Figure 5E). However, *P. indica*-primed, and the uninoculated chilli plants were free from disease symptoms during the experimental period.

Apart from the increased tolerance to the disease, *P. indica* improved shoot and root growth in the primed chilli plants. On the 10th DAI, uninoculated *P. indica*-primed plants displayed maximum root (5.13 g) as well as shoot biomass (5.38 g) compared to non-primed control (3.70 g and 4.31 g, respectively). Irrespective of pathogen inoculation, *P. indica*-colonized plants had increased root and shoot fresh weights of 4.20 g and 4.83 g, respectively, than the control plants (3.26 g and 4.10 g, respectively) (Figures 5F–5G). Thus, we establish that *P. indica* protects primed chilli plants from the incidence and spread of anthracnose caused by *C. capsici*, added with growth and yield benefits.

***P. indica* enhances the phenol content and activities of defense-related enzymes in chilli plants.** *P. indica*-primed chilli plants were more tolerant to anthracnose disease than the control plants. Biochemical analysis of phenols and various defence-related enzymes in the three-party interaction of *C. capsici* and *P. indica*–primed seedlings was considered for the study. Leaf samples from four treatments were collected and analyzed at 0, 12, 24 and 72 HAI.

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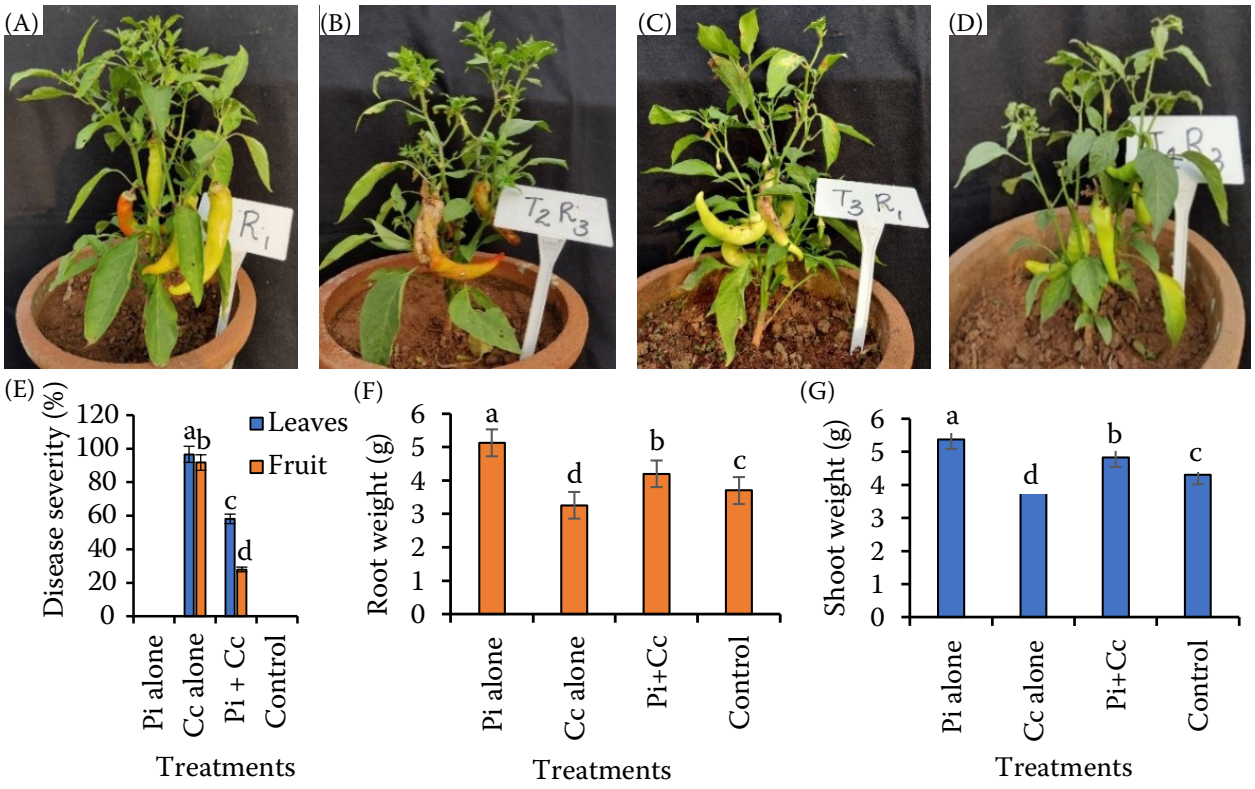

Figure 5. *Piriformospora indica* shields chilli plants against anthracnose infection in pot culture

60-day-old potted chilli plants were inoculated with *C. capsici* and were observed for anthracnose disease symptoms and severity. Four treatment combinations were maintained. (A) chilli plants colonized with *P. indica* (Pi alone); (B) chilli plants inoculated with *C. capsici* alone (Cc alone); (C) *P. indica*-primed chilli plants inoculated with *C. capsici* (Pi + Cc); (D) chilli plants without any treatment (control); (E) the graph represents disease severity (%) in different treatments after *C. capsici* inoculation in 60-day-old chilli plants. Data based on five independent biological replicates and bars represent SEs; (F) fresh root weights and (G) fresh shoot weights of different treatments in 60-day-old chilli plants at 10 DAI. Bars show the analysis result of five independent biological replicates, and different letters above the bars represent significantly different values

Phenolic biosynthesis. Maximum accumulation of total phenolics was noticed in the *P. indica*-colonized treatments. On pathogen inoculation, the total phenolics increased steadily in *P. indica*-primed chilli plants, reaching the highest value of 1.58 mg/g × min at 72 HAI. Total phenolics increased to 1.02 mg/g × min at 24 HAI and then reduced in uninoculated primed plants. Phenolic contents in *C. capsici*-inoculated plants increased gradually towards 72 HAI, which is lower than the values of endophyte co-cultured plants.

The activity of PAL enzyme appeared enhanced in endophyte-treated chilli plants compared to control. Enzyme activity appeared to be high (85.40 µg/g × min) in the endophyte colonized chilli plants inoculated with *C. capsici* at 12 HAI, while the activity in chilli plants primed with *P. indica* alone was enhanced at 24 HAI (70.23 µq/g × min). PAL activity of pathogen-inoculated chilli increased to 12 HAI (63.10 µq/g × min) but later, declined. Chilli plants colonized with *P. indica* exhibited higher CAD activity irrespective of pathogen inoculation at 24 HAI. CAD activity in *C. capsici*-inoculated, *P. indica*-primed plants increased to 9.07 nkat/g (maximum) at 24 HAI while CAD activity was minimum (5.45 nkat/g) in the untreated control. *C. capsici*-inoculated chilli plants showed low CAD enzyme activity of 5.65 nkat/g compared to treatments with *P. indica*. 4-CL activity increased in chilli plants on pathogen inoculation. Maximum activity of 83.07 pkat/mg was recorded in chilli plants inoculated with pathogen alone at 12 HAI. *C. capsici* inoculation in *P. indica*-treated plants increased 4-CL activity at 12 HAI (71.34 pkat/mg), and after that declined. Chilli plants treated with *P. indica* alone recorded the lowest enzyme activity (32.02 pkat/mg).

P. indica-primed seedlings displayed augmented PAL, CAD and PO enzyme activities on inocula-

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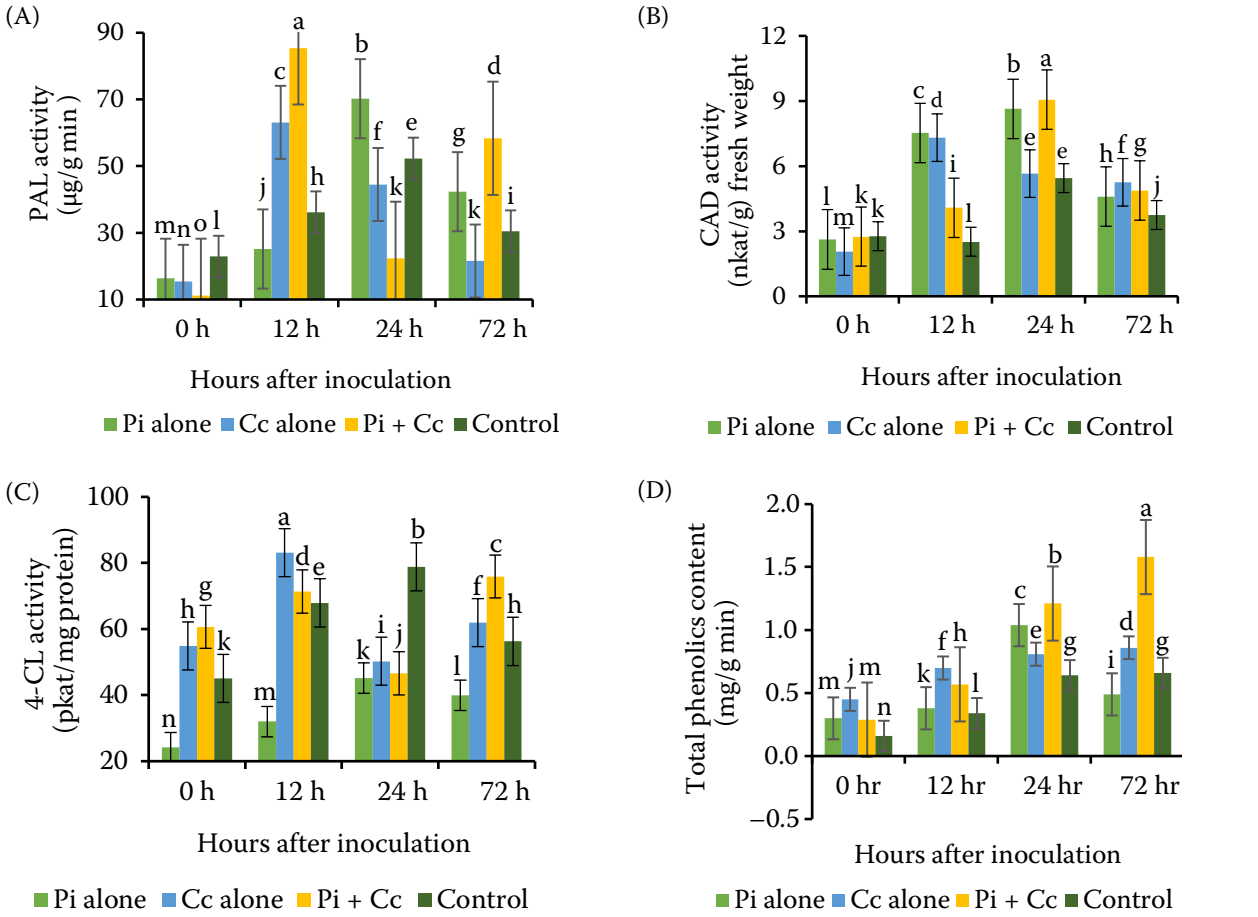

Figure 6. Enzymes involved in phenolic biosynthesis

(A) Phenylalanine ammonia-lyase; (B) cinnamyl alcohol dehydrogenase; (C) 4-coumarylCoA ligase in different treatments at 0, 12, 24 and 72 hours after inoculation of *Colletotrichum capsici*. Fresh leaf samples of chilli variety Vellayani Athulya were collected from four different treatments viz., *Piriformospora indica*-primed chilli plants (Pi alone), seedlings inoculated with *C. capsici* (Cc alone), *P. indica*-primed chilli seedlings inoculated with *C. capsici* (Pi + Cc) and control plants. Bars are based on four independent experiments and represent SEs; (D) total phenolics content

tion with *C. capsici*, with a 25 % increase over the pathogen inoculated control. Maximum enzyme activities were recorded at 12 HAI and declined at 24 HAI. In contrast, higher PPO and 4-CL activities (> 16% increase) were observed in chilli plants exposed to *C. capsici* alone (Figure 6).

Phenolic metabolism. Chilli plants colonized with *P. indica* and inoculated with the pathogen showed maximum PO activity of 84.90 mg/min × g FW at 12 HAI, then reduced towards 24 HAI and again improved at 72 HAI. A similar trend was noticed in *C. capsici* – inoculated chilli plants. Leaves taken from *P. indica*-treated chilli plants showed increased PO activity at 12 HAI (50.32 mg/min × g FW) and later declined. PO activity in control plants showed a gradual increase throughout the timeline. Maximum PPO activityof 6.46 mg/min × g FW was recorded in chilli plants inoculated with pathogen alone at 12 HAI and decreased after that. Minimum activity was observed in control plants at 12 HAI (3.45 mg/min × g FW). On pathogen inoculation, *P. indica*-colonized plants exhibited slow enhancement of PPO activity towards 72 HAI. In comparison, enzyme activities of chilli plants treated with endophyte alone increased at 12 HAI (mg/min × g FW) and then reduced (Figure 7).

DISCUSSION

P. indica, an extensively treasured endophyte, shows an exceptionally broad host range. The fungus is elevated to the level of a universal endophyte

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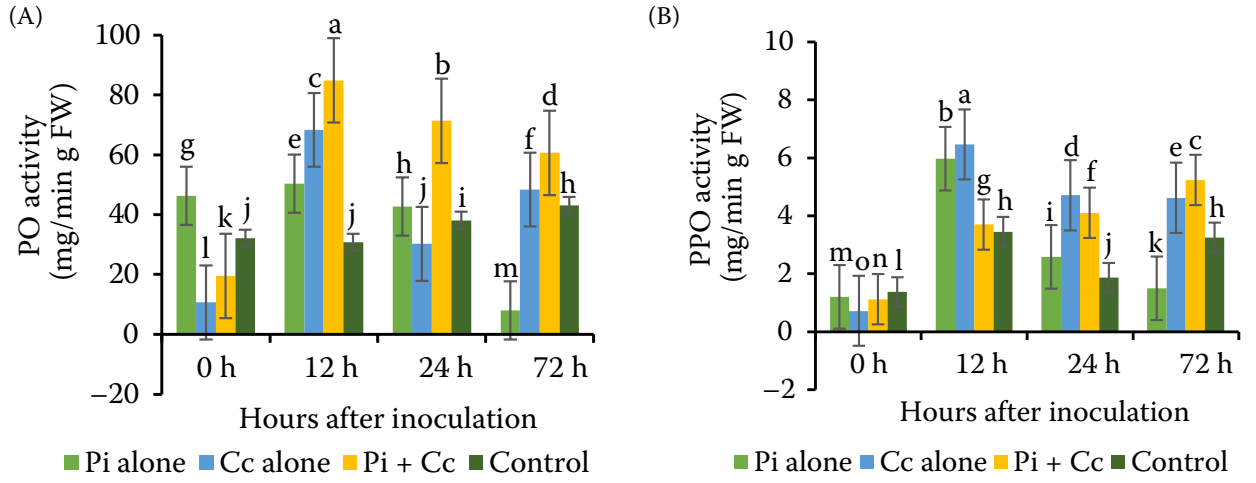

Figure 7. Enzymes involved in phenolic metabolism

(A) Peroxidase; (B) Polyphenol oxidase in different treatments at 0, 12, 24 and 72 hours after inoculation of *Colletotrichum capsici*. Fresh leaf samples of chilli variety Vellayani Athulya were collected from four different treatments viz., *Piriformospora indica*-primed chilli plants (Pi alone), seedlings inoculated with *C. capsici* (Cc alone), *P. indica*-primed chilli seedlings inoculated with *C. capsici* (Pi + Cc) and control plants. Bars are based on four independent experiments and represent SEs

having various growth-promoting and stress-alleviating (biotic and abiotic) effects in the broad group of colonized plants (Franken 2012; Shrivastava & Varma 2014; Mensah et al. 2020). Our present research established a symbiotic endophytic association between *P. indica* and chilli plants, which abetted resistance against chilli anthracnose disease. Waller et al. (2005) suggested the creation of plant antioxidants owing to *P. indica* colonization in host plants, thereby enhancing tolerance to various stresses.

In vitro dual culture experiment depicted that *P. indica* limited growth of the chilli anthracnose pathogen, *C. capsici*. This interaction led to significant boundaries between fungal colonies due to inhibition of further pathogen mycelial growth. Extended storage of these dual culture plates revealed the incursion of inhibition area by *P. indica* followed by thickening and lysis of pathogen hyphae. These results observed were consistent with the formation of sharp, clear borders at the interface site of *P. indica* and take-all pathogen of wheat, *Gaeumannomyces graminis*. This was surpassed by *P. indica*, leading to the lysis of pathogen hyphae on prolonged incubation of culture plates (Serfling et al. 2007; Ghahfarokhi & Goltapeh 2010).

Different conclusions have been drawn from *P. indica* interaction with different plant disease incitants viz., antibiosis, inhibition zone, coiling and choking, overgrowth and sporulation as mutualism or parasitism (Johnson et al. 2013; Ghorbanpour et al. 2018). An inhibition zone was produced in two different experiments involving *P. indica* with two pathogens, *Alternaria brassicae* and *Verticillium dahlia* (Sherameti et al. 2014). *P. indica* restricted mycelial growth and microsclerotia formation in *V. dahlia,* causing wilt disease of Arabidopsis in dual culture. However, no clear inhibition zone was noticed, and the pathogen had zero influence on the endophyte (Sun et al. 2014). Trzewik et al. (2020) detected antibiosis at the interface between post-harvest pathogens (*Aspergillus sydowii* and *Rhizopus stolonifera*) and *P. indica,* whereas restricted growth was noticed in the case of *Aspergillus flavus* and *Aspergillus* sp. Antibiosis was observed in the dual culture experiments between *Rhizoctonia solani* and *P. indica* (Nassimi & Taheri, 2017). Contradictory to the above results, *P. indica* displayed no mycoparasitism against *Fusarium graminearum* (Rabiey & Shaw 2016; Moharam et al. 2017) and *Botrytis cinerea* (Narayan et al. 2017). These studies paved a differential method of action by *P. indica* against various pathogens *in vitro,* indicating the possibility of its utilization as a bio-protectant by enhancing systemic immunity (Oelmüller et al. 2009; Molitor et al. 2011; Johnson et al. 2014).

The decline in size, as well as the number of symptomatic lesions and disease infection rates caused

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by various necrotrophic and biotrophic pathogens in *P. indica*-primed plants, was reported by Johnson et al. (2013). In our *in vitro* and *in vivo* co-cultivation experiments, anthracnose infection due to *C. capsici* was significantly reduced in the endophyte-colonized chilli plants. Days taken for symptom development were also considerably delayed compared to the control plants. This demonstrates the antagonism of *P. indica* against *C. capsici* and the creation of a well-built wall of protection (resistance) in the plant. Kumar et al. (2009) noticed a drastic decrease in the advancement of the disease in *P. indica*-primed maize due to *Fusarium verticilloides*. Previous studies of *P. indica*-treated rice, barley and wheat showed enhanced disease resistance against their respective shoot and root pathogens (Waller et al. 2005; Stein et al. 2008; Rabiey et al. 2015). Endophyte-primed tomato seedlings showed 30% reduction in the disease severity of *V. dahlia* and 20% improvement in leaf biomass (Fakhro et al. 2010). Also, Roylawar et al. (2021) recorded 53.80% (3 days post-infection) and 53.30% (5 days post-infection) lower disease severity in *P. indica*-colonized onion plants on infection with *Stemphylium vesicatoria*, causing onion leaf blight. Khalid et al. (2020) observed a 61.60% reduction in *Plasmodiophora brassicae* infection following *P. indica* co-cultivation. This evidence uncovers the potential of root endophyte, *P. indica,* in controlling a number of plant pathogens and, thus, its application as a biocontrol agent against causal agents of chilli anthracnose. Paul et al. (2021) recorded the lowest lesion size (0.43 cm) and disease severity (10.41%) of *Phytophthora capsici* in black pepper plants on simultaneous inoculation of *P. indica* and *Rhizobium radiobacter* PCRE10.

Sporulation in *C. capsici* was delayed, and no sporulation was observed in the dual culture plates. In the *in vivo* study, primed chilli plants recorded lower anthracnose incidence and severity than non-primed plants. This reduced disease spread may be attributed to limited *C. capsici* sporulation in the presence of *P. indica* and its direct antagonism to the pathogen.

Better root colonization and nutrient exchange leading to extensive variations in root architecture was initiated by endophytes (Lee et al. 2011). Reports on stimulation of root growth improvements with enhanced lateral as well as root hair development were found in *P. indica*-colonized plants by many authors (Varma et al. 1999; Vadassery et al. 2008; Johnson & Oelmüller, 2009; Fakhro et al. 2010; Lahrmann & Zuccaro, 2012; Prasad et al. 2013; Venus & Oelmüller, 2013; Vahabi et al. 2015). Vyshakhi and Anith (2021) co-cultivated chilli, tomato and eggplant with *P. indica* and recorded 41, 83 and 43% of root colonization. Higher secondary and tertiary root formation in *P. indica*-colonised plants helps to mobilize, absorb and translocate more nutrients from soil to plant to maintain plant health, reducing the incidence and severity of the disease. Our studies also demonstrated endophyte-associated biomass enhancement in chilli. This was consistent with the results observed in *Oryza sativa* (Prajapati et al. 2008), *Zea mays* (Zamani et al. 2016), *Medicago truncatula* (Li et al. 2017), *Arabidopsis thaliana* (Abdelaziz et al. 2017), sweet potato (Li et al. 2021), *Brassica napus* (Su et al. 2017), and *Triticum aestivum* (Rabiey et al. 2015; Hosseini et al. 2017). Nandana and Anith (2020) reported the highest root fresh and dry weights in response to combined inoculation of tomato plants with *P. indica* and *Pseudomonas fluorescens* PN026. Similarly, combined inoculation of *P. indica* and *Rhizobium radiobacter* PCRE10 caused enhancement in the growth parameters of bush pepper (Paul et al. 2021).

Colonization of chilli plants with the endophyte strongly stimulated the increase in total phenols. Total phenols were comparatively enhanced in the primed plants than control at 12 HAI. Sharma et al. (2017) noticed that primed *Aloe vera* plants recorded a 147.30% increase in phenol accumulation. Similarly, phenolic acids increased significantly in the fungus-colonized *Artemisia annua* plants (Khalid et al. 2020). Endophytic colonization induced a considerable increase in phenol contents and the contribution of various defence enzymes involved in the phenylpropanoid pathway. Higher enzyme activities of PAL, PO, and CAD were observed at 12 HAI in the endophyte-treated plants, which later decreased to 24 HAI and again increased to 72 HAI. This trend may be correlated with early resistance induction by *P. indica* against *C. capsici*. In contrast, the activities of PPO and 4-CL were increased in pathogen-inoculated plants. The plant's innate defence mechanism may be the reason for increased induction, which further declined on disease progression. Plants have an inherent capacity to detect their pathogens by means of certain pathogen elicitors, known as microbe-associated molecular patterns (MAMPs). Contact with pathogenic or non-pathogenic microorganisms induces

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oxidative burst ion flux followed by protein phosphorylation or dephosphorylation, producing defence molecules (salicylic acid, jasmonic acid, ethylene, antioxidants). This further stimulates the production of enzymes and the expression of various genes involved in plant defence. Vanitha et al. (2009) observed increased enzyme activity and accumulation of phenolics in resistant plants inoculated with pathogens. Plants defend against pathogens by lignification and suberization of cell walls followed by induction of defence-related enzymes (peroxidase, phenylalanine ammonia-lyase and polyphenol oxidase) (Prasannath 2017).

C. capsici inoculation in *P. indica*-primed plants caused a steady surge in activities of PPO and 4-CL enzymes 0–72 HAI. *P. indica* induced accelerated levels of PAL activity, increasing 1.37-fold at 24 HAI (Tashackori et al. 2016). Lava et al. (2021) recorded maximum PO and PPO enzyme activities in *P. indica*-primed cucumber plants against *F. oxysporum*, causing Fusarium wilt. Endophyte-treated gerbera plants, inoculated with *Phytophthora cryptogea* showed higher activities of PAL and PO enzymes (Wu et al. 2022). Similarly, *P. indica* pre-inoculation in wheat against *Rhizoctonia cerealis* and *F. graminearum* led to increased PO and PPO enzyme activities (Li et al. 2022). *Scrophularia striata* cell cultures added with culture filtrate from *P. indica* displayed enhanced activities of PAL and CAD enzymes (Shahkarami et al. 2022).

Root endophyte, *P. indica* can provide resistance against both root and shoot diseases. Root endophytes involved in biocontrol are usually associated with root disease incitants, but *P. indica* also provides resistance against shoot pathogens (Waller et al. 2005). Kumar et al. (2009) observed increased enzyme activities in *P. indica*-primed maize plants inoculated with *Fusarium verticilloides*. The endophyte reduced the incidence and spread of tomato wilt (Sarma et al. 2011) and leaf curl (Wang et al. 2015) diseases. *V. dahliae* infection was significantly lowered in the *P. indica*-primed Arabidopsis plants than in untreated control (Sun et al. 2014).

Colonization by endophytes stimulates antioxidants and plant hormones and up-regulation of defense genes to reduce foliar infection in plant hosts. On pathogen attack, *P. indica* increases the production of antioxidant enzymes that scavenge reactive oxygen species (ROS) to counteract pathogen invasion, thus mitigating stress. Endophyte also induces the release of various Phyto-hormones viz. gibberellins, ethylene, brassinosteroids, abscisic acid and cytokinin within colonized hosts (Schäfer et al. 2009). Genomic studies of *P. indica*-primed plants revealed increased response of several defensive genes viz. Ethylene Response Factor1 (*ERF1*), Variable small protein (*VSP*), Plant defensin1.2 (*PDF1.2*), Lipoxygenase2 (*LOX2*), non-expression of pathogenesis-related gene1 (*NPR1*) which subsequently triggered defence reactions against the pathogen (Khalid et al. 2019).

Mutualism in plants helps enhance nutrient supply, increase growth, and improve tolerance and resistance against drought, salinity, pathogens, and herbivore attacks (Rodriguez & Redman 2008). *P. indica* also induces systemic resistance (ISR) in *Arabidopsis* by stimulating cytosolic and not nuclear-localized *NPR1* (Stein et al. 2008). In *P. indica* colonized plants, many pathogenesis-related genes, mainly ethylene signalling genes (*ERF1*) and jasmonic acid signalling genes (*VSP*, *PDF1.2*, *LOX2*), were up-regulated on exposure to pathogens (Molitor et al. 2011). Increased expression of defence and pathogen-related genes were noticed during early interaction and but suppressed in later stages of interaction (Wang et al. 2014).

CONCLUSION

In summary, our results demonstrate that *P. indica* is an efficient biocontrol fungus in controlling chilli anthracnose, as instigated by *C. capsici*. This endophyte can aid in strengthening chilli plants against further pathogen infection by stimulating the defence enzymes. Future research is to focus on studies related to various signalling pathways, different plant hormone levels, and defence gene expressions, as well as the relationship between various components involved in the tripartite interaction.

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Received: November 15, 2023
Accepted: May 7, 2024
Published online: September 6, 2024