Fungal root endophyte *Piriformospora indica* is compatible with strobilurin fungicides

Abstract

Colletotrichum gloeosporiodes causing anthracnose is an important fungal disease of yard long bean infecting leaf, stem, petiole, flower and pod leading to significant yield loss. New generation fungicides *viz.* strobilurins and triazoles are widely used in the management of the disease. *Piriformospora indica* is a widely used beneficial root endophytic fungus that suppresses plant diseases in addition to enhanced growth promotion. The present study was outlined in completely randomized design (CRD) to test the compatibility of *P. indica* with the new generation fungicides by poison food technique in petri dishes and broth media and by calculating the percentage of conidial germination. The results revealed that *P. indica* was compatible with strobilurins, combination fungicides of strobilurins and triazoles, carbendazim and pencycuron upto 90 per cent till 350 ppm in poison food and broth experiments. Moreover, germination of the chlamydospores was significant in number in these fungicides. But, triazole fungicides completely inhibited the mycelial growth and spore germination of *P. indica*Thus, *P. indica* is compatible with strobilurins and combination fungicides.

Keywords: *P. indica*, root endophyte, fungicides, compatibility, fungicide residue, dissipation

Introduction

Yard long bean (*Vigna ungiculata* subsp. *sesquipedalis* (L) Verdcort) is a highly preferred protein rich vegetable with many health benefits. In Kerala it is grown for its long and succulent green pods.The crop is severely affected by fusarium wilt, viral diseases, anthracnose and cercospora leaf spot; among which, anthracnose caused by *Colletotrichum gloeosporioides* is one of the most important fungal disease (Emechebe and Lagoke, 2002), causing an yield loss of 50 per cent (Kumar, 1999). The disease is widely distributed in the Asian and African continents. On susceptible cultivars, typical anthracnose lesions (brown to tan, sunken, and lenticular) quickly grow and consolidate to girdle stems, peduncles, and petioles (Sreeja, 2014). High susceptibility of the disease is due to the lack of genetic resistance, epidemiological conditions, cultivation practices and the emergence of fungicide-resistant strains of the pathogen. Management of the disease is currently carried out by spraying triazole fungicides, and the combination fungicide carbendazim and mancozeb (Deeksha and Tripathi, 2002; Kulkarni, 2009; Sreeja *et al.* 2015) and biocontrol agents like *Trichoderma* spp. (Sreeja, 2014).

Application of the fungicides against fungal infections is regarded as a common method to secure regular food supply (Strange and Scott, 2005). According to preliminary statistics of the U.S. Geological Survey (USGS), dithiocarbamates, triazoles, strobilurins and its combination fungicides constitutes the major fungicide category among synthetic fungicides. These chemicals are highly efficient in action, but their rigorous use can damage the environment and pose severe health hazards. In this context, the use of biocontrol agents is highly influential against plant diseases and can reduce chemical fungicide usage and its impending health hazards.

Endophytes, which inhabit the plant tissue, possess all the properties of biocontrol agents as they shield the plant throughout its life stages. They are now a viable substitute to agrochemicals. According to Cook and Baker (1983), a mixed formulation of chemicals and biological control antagonists can extend the duration and activate disease control, which can reduce the quantity of chemical; residue buildup, lessen pathogen virulence and make it more prone to attack by antagonists (Lorito *et al*., 1996). *Piriformospora indica* is one such fungus that is axenically culturable, invades broad hosts, is root colonizable and is mycorrhiza like versatile endophytic fungus that helps in better plant growth and performance (Varma *et al.*, 2012). *P. indica* is a potent bioregulator as it enhances shoot and root proliferation by synthesizing phytohormones, a good biofertilizer that gives multifaceted responses against various plant diseases (Waller *et al*., 2005; Deshmukh *et al*., 2006; Kumar *et al*., 2009; Yadav *et al*., 2010; Dolatabadi *et al*., 2011; Johnson *et al*., 2014; Gill *et al.,* 2016).

The compatible nature of strobilurin fungicides and carbendazim with *Trichoderma* spp., *Pseudomonas* Spp. and *Bacillus subtilis* was demonstrated by Sendhilvel *et al*. (2004), Anand *et al.* (2007), Archana *et al*. (2012), Bagwan (2010), Sarkar *et al.* (2010); Ranganathswamy *et al*. (2012), whereas triazole fungicides were inhibitory to the bio-agents.

It is our first effort to investigate the compatibility of strobilurin, triazole and its combination fungicides with *P. indica*. Henceforth, we attempted to find the compatible new generation fungicides with *P. indica* under *in vitro* conditions under petri plate, broth and via chlamydopsore germination.

Materials and methods

Maintenance and multiplication of *P. indica*

P. indica (Accession No. INBA3202001787) was cultured in Potato Dextrose Agar (PDA) medium (pH-6.5). The culture was maintained at Department of Plant Pathology, College of Agriculture, Vellayani. Five mm mycelial discs were cut out from the actively growing regions of hyphae and placed at the middle of petri dishes. The plates were incubated at room temperature (27±1°C) and at 80 per cent humidity with 12 hour dark and light for 10 days. It was sub-cultured once in fifteen days for its maintenance (Johnson *et al.,* 2013). Periodical re-isolation was carried out from the colonized roots to maintain the colonization efficiency of the endophyte. For further multiplication, *P. indica* was cultured in Potato Dextrose Broth (PDB) (pH-6.5) in conical flasks and incubated at 70 rpm for 21 days at 27 °C to produce a sizable amount of mycelial mat.

Compatibility assay

New generation systemic fungicides *viz.*, three strobilurin (trifloxystrobin 50 WG, kresoxym-methyl 44.3 SC, azoxystrobin 23Sc), four triazole (hexaconazole 5 EC, difenoconazole 25% EC, propiconazole 25% EC, tebuconazole 25.9% EC), two combination (azoxystrobin 11 % + tebuconazole 18.3 % SC, trifloxystrobin 25 % + tebuconazole 18.3 % SC) fungicides, a contact fungicide (pencycuron 22.9 % SC) and systemic fungicide (carbendazim 50WP – positive control) at 100, 250, 350, 500 and 1000 ppm concentrations were tested *in vitro* against *P. indica* by recording the radial mycelial growth and nature of mycelial growth at various time intervals.

Double strength PDA (50ml) and sterile distilled water (50ml) was prepared in 250ml conical flasks and sterilized. *In vitro* compatibility of *P. indica* with the fungicides was determined by preparing desired concentrations of chemicals in sterile water and dispersing it with molten double strength PDA aseptically. *P. indica* was cultured in poisoned-PDA plates and a mycelial plug of 5mm diameter was placed at the centre of the Petri plates. PDA medium without fungicide served as control. Each treatment was replicated thrice. Observations of mycelial growth were recorded after 1, 3, 5, 7, 10 and 15 days after incubation along with the days taken for full growth of *P. indica*. The per cent inhibition of *P. indica* by each fungicide treatment was calculated using the formula suggested by Vincent (1947).

$$\text{Per cent inhibition of growth} = \frac{C-T}{C} \times 100$$

C= Growth of *P. indica* in control (mm)

T= Growth of *P. indica* in treatment (mm)

One hundred ml PDB and 100 ml sterile water was prepared in 250ml conical flasks and sterilized. 10000 ppm concentration of stock solution of each fungicides were prepared in 100 ml sterile water and the concentration was diluted to 100, 250, 350, 500 and 1000 ppm by pipetting required amount from stock to each of the PDB containing flasks. 5 mm disc of *P. indica* culture was added to each conical flasks having different concentration of the fungicides. Control flasks were also maintained. The wet and dry weight of *P. indica* mycelium was recorded at 21 days of growthalong with the control.

Spore germination inhibition study

P. indica was cultivated in 100ml of PDB for 21 days under room temperature at 70 rpm for ample chlamydopsore production and the pores were mixed with sterile water.Stock solutions of the fungicides were prepared at concentrations 100, 250, 350, 500 and 1000 ppm. Sterilized cavity slide and cover slips were used in the study. Each cavity slide had 50 µl of each fungicide concentration and 50 µl of *P. indica* spore suspension (10^{-6} ml^{-1}), thoroughly mixed, and incubated at room temperature. Germination of spores was recorded and per cent inhibition was calculated for each fungicide at different intervals.

Statistics

“The Kerala Agricultural University's GRAPES, (General R-shiny Based Analysis Platform Empowered by Statistics; https://www. kaugrapes.com/home) a R-based analysis platform was used for the statistical analysis. Using Duncan's Multiple Range (DMRT) and one-way Analysis of Variance (ANOVA), the treatment means were compared with a probability of 0.05 per cent level of significance. All data shown are mean ± standard deviation (SD) of at least three biological replicates” (Reshna *et al.*, 2022).

Results

Compatibility of systemic fungicides with *P. indica* under *in vitro*

The data presented in the Table 2 demonstrated the compatibility of *P. indica* with three strobilurin fungicides at different time intervals (3rd, 5th, 7th, 10th and 15th day). A minimal inhibition of 1.12 per cent was observed with trifloxystrobin 50WG at 100ppm after 10 days of incubation (Fig. 1a). However, an inhibition of 21.22 and 25.56 per cent respectively was noticed at 250 and 350ppm on 10th day. Azoxystrobin (Fig. 1b) and kresoxym-methyl 44.3 SC (Fig. 1c) were less toxic to and compatible with *P. indica* at all concentrations tested. Thus all three strobilurin fungicides were compatible with *P. indica*. Pencycuron 22.9% SC (Fig. 1d) also recorded a compatibility of 88.88, 76.66 and 56.67 per cent at 100, 250 and 350 ppm concentration respectively (Fig. 2).

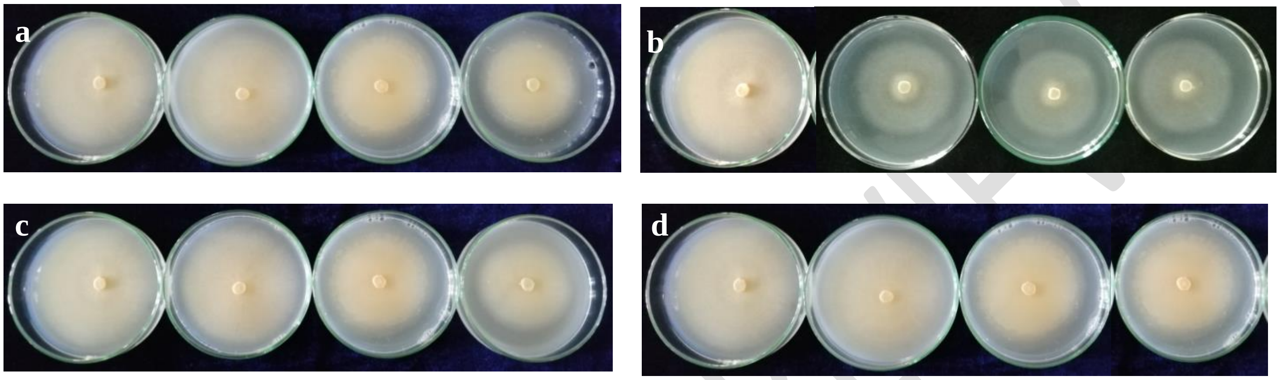

Figure 1: Compatibility of a) Trifloxystrobin b) Azoxystrobin c) Kresoxym methyl d) Pencycuron with *P. indica* at 100, 250 and 350 ppm concentrations. *P. indica* is grown on PDA amended with respective fungicides and mycelial growth was recorded at various time intervals

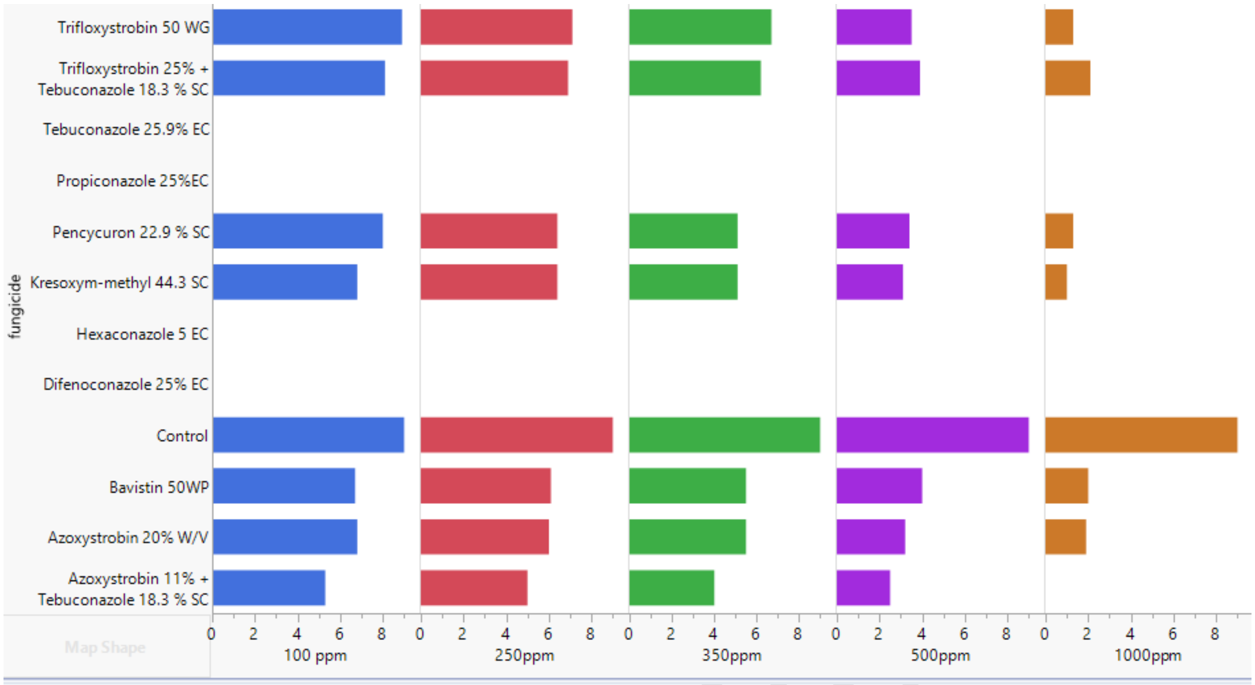

Figure 2: Mycelial growth *P. indica* at different concentrations 100, 250, 350, 500 and 1000 ppm of fungicides. *P. indica* is grown over PDA amended with respective fungicides and mycelial growth was recorded

The combination fungicide, trifloxystrobin 25 % + tebuconazole 18.3 % SC at 100, 250 and 350 ppm recorded a compatibility of 90.00, 76.67 and 68.89 per cent with *P. indica* (Fig. 3a). On the contrary, azoxystrobin 11 % + tebuconazole 18.3 % SC (Fig. 3g) and carbendazim 50 WP showed moderate compatibility of 41.11 to 55.56 per cent and 61.11 to 74.44 per cent with the endophyte (Fig. 3b). However triazole fungicides, hexaconazole 5% EC, propiconazole 25%EC, tebuconazole 25.9% EC and difenoconazole 25% EC (Fig. 3c, 3d, 3e, 3f) were highly inhibitory to *P. indica* with cent per cent inhibition. No differences were observed in the nature of mycelial growth when *P. indica* was grown together with compatible fungicides.

Compatibility of fungicides with *P. indica* was tested in poisoned PDB. Mycelial weight of *P. indica* was recorded on 21st day in control as well as treated broth cultures. Fresh mycelial weight of untreated control flasks were 12g and significant reduction in weight was observed with different fungicide concentrations (Fig. 4). Triazole fungicides *viz.*, hexaconazole 5 EC, difenoconazole 25% EC, propiconazole 25% EC and tebuconazole 25.9% EC showed complete inhibition of mycelial growth of *P. indica* at all the concentrations (Table 1). The mycelial growth was significantly higher when grown along with strobilurin fungicides, carbendazim and pencycuron, showing a compatible reaction. Fresh mycelial weight of the endophyte was ranging from 1.1 to 1.6 g for these fungicides at different concentrations.Combination fungicides also depicted a compatible reaction. No differences in nature of mycelial growth were observed in all the fungicides tested; but its varying growth pattern was noticed. 5 to 6 times reduction in weight of mycelia was noticed when it was dried.

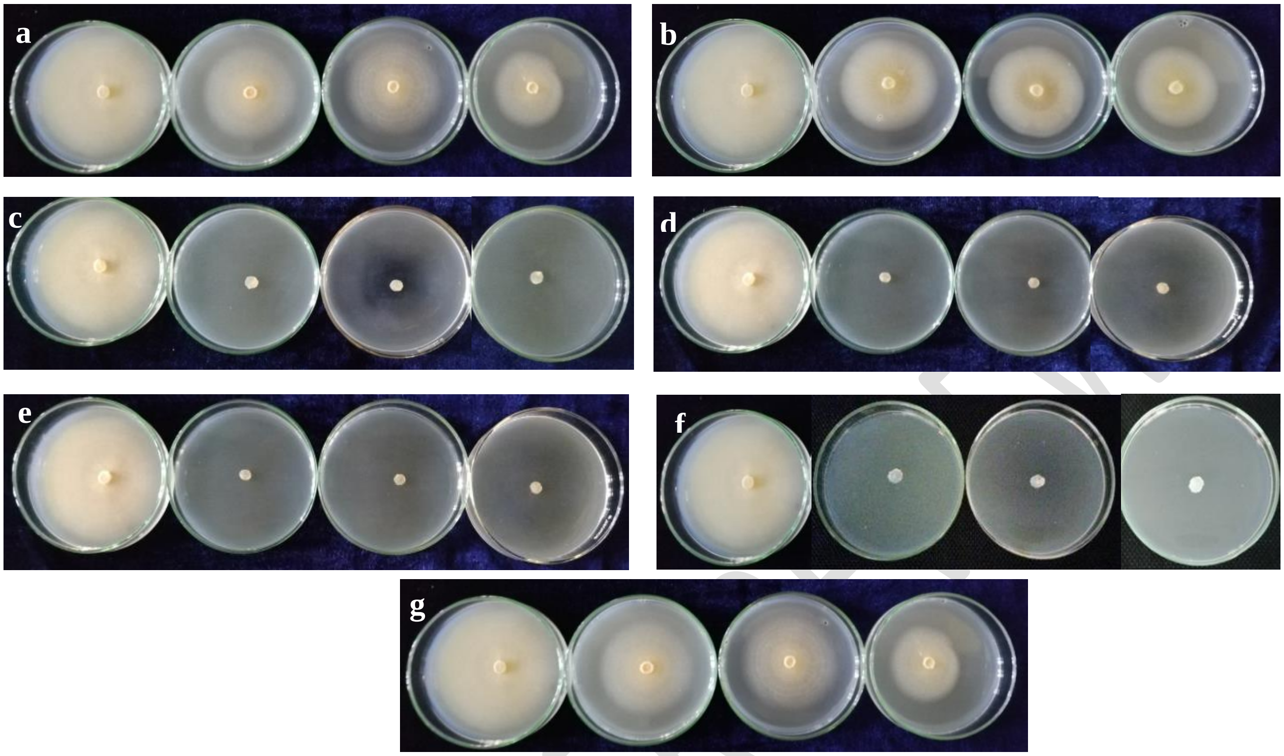

Figure 3: Compatibility of a) Trifloxystrobin 25 % + Tebuconazole 18.3 % SC b) Carbendazim 50 WP c) Hexaconazole 5 EC d) Propiconazole 25 %EC e) Tebuconazole 25.9% EC f) Difenoconazole 25% EC g) Azoxystrobin 11 % + Tebuconazole 18.3 % SC against *P. indica* at 100, 250 and 350 ppm concentrations. *P. indica* is grown over PDA amended with respective fungicides and mycelial growth was recorded.

Figure 4: Compatibility of a) Trifloxystrobin 25 % + Tebuconazole 18.3 % SC b) Pencycuron e) Kresoxym methyl d) Trifloxystrobin e) Azoxystrobin f) Carbendazim 50 WP g) Azoxystrobin 11 % + Tebuconazole 18.3 % SC against *P. indica* at 100, 250 and 350 ppm concentrations. *P. indica* is grown over PDB amended with respective fungicides and mycelial growth is recorded.

Table 1: Mycelial weight of *P. indica* with fungicides *in vitro* in PDB

Treatments (Fungicides)	Mycelial growth (cm) on 10th day									
	100ppm		250 ppm		350 ppm		500 ppm		1000 ppm	
	F	D	F	D	F	D	F	D	F	D
Trifloxystrobin 50 WG	11±0.86	2.2±0.04	9.46±0.05	1.8±0.12	8.93±0.12	1.78±0.05	4.6±0.04	0.92±0.07	1.73±0.03	0.34±0.02
Kresoxym methyl 44.3 SC	9.1±0.03	1.81±0.05	8.53±0.03	1.7±0.03	6.8±0.01	1.36±0.03	4.13±0.01	0.82±0.03	1.6±0.03	0.32±0.03
Hexaconazole 5 EC	0	0	0	0	0	0	0	0	0	0
Difenoconazole 25% EC	0	0	0	0	0	0	0	0	0	0
Pencycuron 22.9 % SC	10.7±0.18	2.13±0.07	8.53±0.14	1.7±0.02	6.8 ±0.01	1.36±0.02	4.53±0.06	0.9±0.02	1.73±0.01	0.34±0.01
Azoxystrobin 11% +Tebuconazole 18.3 % SC	9.7±0.11	1.7±0.08	8.3±0.04	1.5±0.02	6.2±0.02	0.99±0.07	2.56±0.02	0.51±0.03	0	0
Trifloxystrobin 25%+ Tebuconazole 18.3 % SC	10.8±0.17	2.16±0.08	9.2±0.11	1.8±0.02	8.26±0.01	1.65±0.02	5.2±0.04	1.04±0.02	2.1±0.03	0.56±0.02
Propiconazole 25% EC	0	0	0	0	0	0	0	0	0	0
Bavistin 50WP	8.93±0.08	1.78±0.02	8.13±0.02	1.6±0.01	7.33±0.07	1.46±0.02	5.33±0.14	1.06±0.01	2.67±0.06	0.52±0.03
Azoxystrobin 20% W/V	9.08±0.06	1.81±0.01	8.0±0.02	1.6±0.08	7.32±0.04	1.45±0.02	4.26±0.02	0.85±0.02	2.53±0.03	0.50±0.03
Tebuconazole 25.9% EC	0	0	0	0	0	0	0	0	0	0
Control	12.1±0.22	2.35±0.04	11.9±0.17	2.3±0.03	11.8±0.21	2.35±0.04	11.7±0.17	2.2±0.03	11.8±0.21	2.4±0.02
SEm±	0.000		0.000		0.000		0.010		0.000	
CD(0.05)	0.036		0.013		0.017		0.172		0.008	

Spore germination inhibition study

Percentage of chlamydospores germinated was calculated after growing *P. indica* with new generation fungicides. Germination of chlamydospores was completely inhibited by four triazole fungicides. However the germination was approximately 60-70 per cent with trifloxystrobin 50 WG, kresoxym-methyl 44.3 SC, azoxystrobin 23SC, pencycuron 22.9% SC and carbendazim 50WP. Combination fungicides also recorded less inhibition in germination of chlamydospores (Fig. 5.).

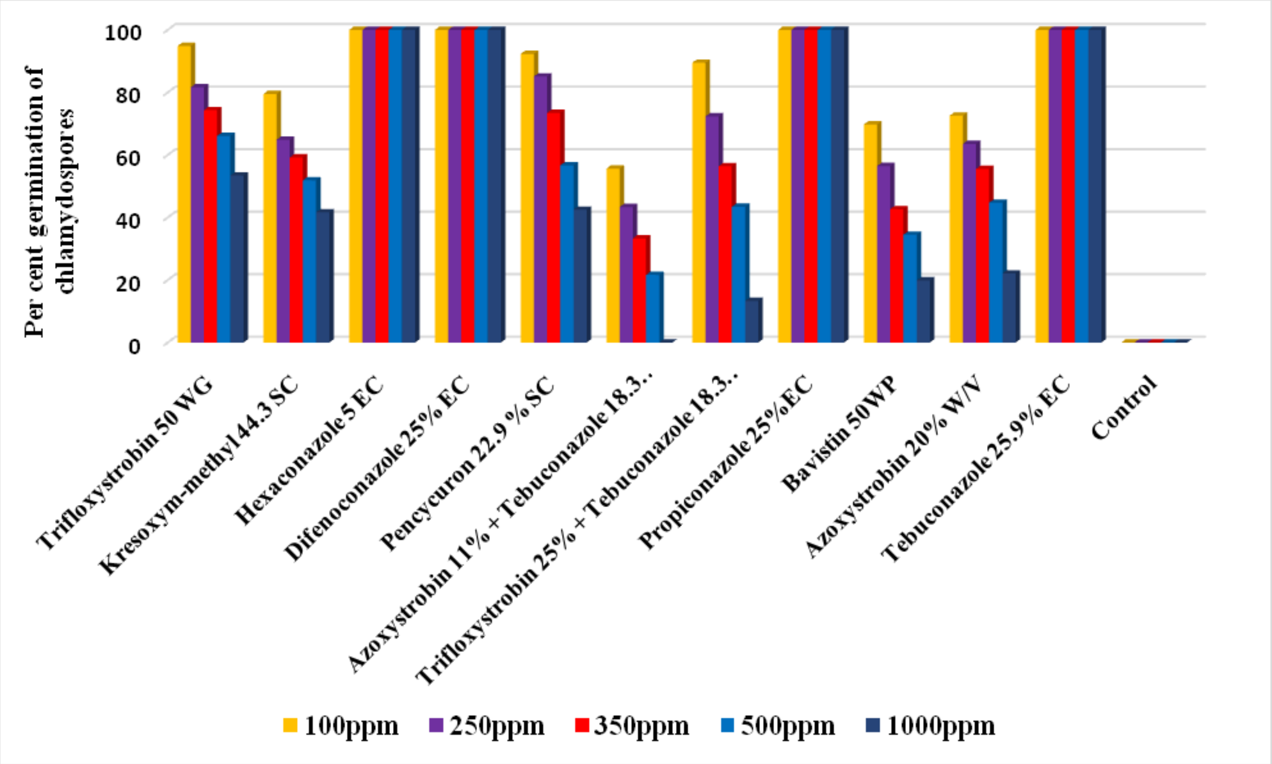

Figure 5: Effect of fungicides on spore germination of *P. indica.* Percentage of chlamydospores germinated was recorded after allowing the spore suspension to grown against different doses of fungicides

Discussion

Use of agrochemicals can never be replaced in agriculture and their impending effects on environment and health should be addressed. Before implementing the usage of endophytic agents in the field, the efficacy of pesticides used in controlling pests and diseases against the particular targeted crop should be determined. It is necessary to understand the compatibility of chemicals with endophytic agents to formulate a better integrated management strategy.

Strobilurins and combination fungicides were most compatible with *P. indica*. The germination of chlamydospores and mycelial growth of *P. indica* were not inhibited by the strobilurins and its combination fungicides. Strobilurin is a natural compound isolated from the mushroom *Strobilurus tenacellus* (Anke, 1977) and contains a toxiphoric (E)-β-methoxyacrylate group (Balba, 2007). It inhibits the mitochondrial respiration of fungi by binding to the quinol oxidation (Qo) site of cytochrome bc1 (Feng *et al.*, 2020). However, the fungicide did not act at the target site of *P. indica*. Similarly, Sendhilvel *et al*. (2004), Anand *et al.* (2007), Archana *et al*. (2012), Louis *et al*.(2016), Suneeta *et al*. (2017), Hanuman and Madhavi (2018), Sharma *et al*. (2017), Widmer (2019), Maheswary *et al*. (2020); Sanchez-Montesinos *et al*. (2021) also reported the compatibility of strobilurin with *Trichoderma* spp., a widely used biocontrol agent.

P. indica was found compatible with pencycuron 22.9 % SC at all concentrations (Fig. 1d) with reduced compatibility at very high concentration of 1000 ppm. Pencycuron, a non-systemic phenyl urea fungicide, destroys the microtubules of fungus and changes the osmotic stability and fluidity of the plasma membrane (Ueyama and Kurahashi, 2007). Madhavi *et al*. (2011), Elshahawy *et al*. (2016), Silva *et al.* (2018); Sanchez-Montesinos *et al*. (2021) also reported a similar compatible action against *Trichoderma* spp.

All four triazole fungicides (Fig 1b and f) completely inhibited the mycelial growth of *P. indica* and germination of chlamydopsores. Triazole fungicides as demethylation inhibitors act on the enzyme C14-demethylase that plays a major role in sterols production, which are essential molecules that ensure stability in the lipid layer (Dupont *et al*., 2011). Similarly, Sarkar *et al.* (2010), Madhusudhanan *et al*. (2010), Madhavi *et al*. (2011), Sreeja and Girija (2015), Sonavane and Venkataravanappa (2017); Maheswary *et al*. (2020) reported complete mycelial inhibition of *Trichoderma* spp. with hexaconazole, difenoconazole and propiconazole as it was found that the fungicide completely inhibits the sterol production of the endophyte.

Conclusion

From the present investigation it was found that strobilurin, its combination fungicide and pencycuron are highly compatible with fungal endophyte *P. indica*. Fungicides can be used in *P. indica*-colonized yard long bean plants to protect the plants from anthracnose infection. This method can also reduce the concentration of chemical used and also reduces the buildup of residue on the plant surface thereby reduce health and environmental risks.

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