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Efficacy of bio control agents for management of *Phytophthora megasperma* causes of collar rot of peas

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Abstract

Pea (*Pisum sativum* L.) is a popular pulse crop in the world. It is attacked by various biotic diseases but collar rot caused by *Phytophthora megasperma* occurs in severe form and causes colossal losses every year. At present this disease is controlled by application of fungicides. But due to serious health hazards of pesticides and environmental concerns, non-chemical approaches are preferred these days. Bio control agents are considered safe and effective to manage soil borne plant diseases. In current work, three characterized *Trichoderma* isolates viz. *Trichoderma harzianum*, *T. viride* and *T. asperellum* and one isolate of *Bacillus subtilis* were testedagainst the pathogen *P. megasperma* by using different antagonistic assays i.e. dual culture, volatile metabolites and nonvolatile metabolites. All the treatments effectively reduced the radial growth of the pathogen during dual culture assay. Nonvolatile metabolites displayed higher growth inhibition of the pathogen as compared to volatile metabolites. *T. asperellum* showed maximum inhibition of the pathogen (47.60%) while minimum inhibition was shown by *T. viride* (26.90%). Present studies conclude that biocontrol agents can be successfully used to manage soil borne plant diseases with no adverse effects or health hazards. These studies will pave the way for effective eco-friendly management of plant diseases in future.

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Introduction

Pea (*Pisum sativum*) is an important winter vegetable crop which is cultivated in Pakistan, India, and America (Majid *et al*., 1996). In Pakistan, it is cultivated on large agro ecological areas including Jhang, Sahiwal, Sheikhupura and Okara. Different biotic and abiotic factors directly influence its production. Pea crop is attacked by many diseases but collar rot caused by *Phytophthora megasperma* is very devastating disease (Mustafa *et al*., 2017). Common symptoms of this disease include root rotting and rot near collar region. In severe attack, plant shows wilting and later die in short time. If pathogen attacks at pod formation time, then poor grains are formed which directly affect its yield. *P. megasperma* attacks a wide range of plant species like berseem, lucerne, chickpea, soybean and mung bean. Pesticides are currently used to manage this disease but continuous use of these chemicals creates resistance in pathogen and causesenvironmental pollution and serioushealth hazards. Now researchers are turning to biological management of hytopathogens. *Bacillus* and *Trichoderma*spp are being used against soil borne pathogens especially against *Phytophthora* species (Wang and Xaio, 2013; Keswani *et al*., 2014). *Trichoderma* spp. has different interactional mechanisms like mycoparasitism, competition and production of antibiotics to antagonize the phytopathogens (Pal and Gardener, 2006). Many species of *Trichoderma* isolates produce different volatile and nonvolatile metabolites which are lethal to phytopathogens (Qualhato *et al*., 2013). Bacterial isolates have also proved their antagonistic ability against phtopathogens. *Bacillus* and *Pseudomonas* were antagonistic to *Phytophthora drechsleri* and *P. capsici* (Singh and Dubey, 2010). Present study was performed with the objective to evaluate different biocontrol agents against *P. megasperma* under laboratory conditions and select the best ones for field use.

Materials and methods

Samples showing diseased symptoms near collar regions were collected from pea fields located in Jhang district, Punjab, Pakistan. Samples were washed with tap water to remove soil particles. Diseased samples were disinfected with 1% NaOCl for three minutes followed by three washings with distilled water. Samples were cut into small pieces and isolation was performed on PARP medium (corn meal agar 17g L^{-1}, 0.4ml Pimaricin L^{-1}, 0.25g Ampicilline L^{-1}, 0.01g Rifampcin L^{-1}, 5mL Pentachloronitrobenzene (PCNB) L^{-1}, 1mL Dimethyl sulphooxide (DMSO) L^{-1}) under aseptic conditions. Pathogen was identified using morphological and cultural characteristics (Bush *et al*., 2006). Culture was maintained on PDA medium for future use.

Trichoderma culture

Three molecularly characterized isolates of *Trichoderma*n amely *Trichoderma asperellum*, *T. harzianum* and *T. viride* were taken from Plant Pathology laboratory, College of Agriculture, University of Sargodha.

Bacterial culture

Soil samples from chili field were collected and their isolation was done by using serial dilution technique (Maleki *et al*., 2011). Fifty µl solutions was spread on PDA plates and incubated at 25±1°C for 36 hours (Maleki *et al*., 2011). Identification was performed on the basis of morphological and cultural characters as described by Schaad *et al*., (2001).

Antagonistic Activity

Dual culture

For Trichoderma isolates

Five days old, 6mm mycelial plugs of pathogen and antagonist were placed opposite to each other aseptically on petri plates which contained sterilized PDA medium and sealed with parafilm.

For Bacillus isolate

Five days old, 6mm mycelial plug of pathogen was placed at one side of sterilized PDA plate, then a loop full of two days old culture of *Bacillus* was streaked out on the opposite side of mycelial plug of pathogen and sealed with parafilm and incubated at 24±1°C. Petri plate containing only pathogen was served as control.

Metabolites

Volatile metabolites

To evaluate/check the antifungal activity of volatiles metabolites of *Trichoderma* against *P. megasperma*, technique described by Raza *et al.*, (2013) was used. Five days old, 6mm mycelial plugs of *Trichoderma* isolates and *P. megasperma* were placed at center of PDA petri plates aseptically. Plates containing *P. megasperma* were placed over on *Trichoderma* containing plates for three days. All petri plates were sealed with parafilm and incubated at 25±1°C. Same procedure was used for volatile metabolites of bacillus isolates.

Culture filtrate / Nonvolatile metabolites

For Bacillus

To evaluate/check the antifungal activity of culture filtrates of two *Bacillus* isolates against *P. megasperma*, technique described by (Abdul Kareem *et al.*, 2014) was used. A 6mm plug of *B. subtilis* was inoculated into 100ml nutrient broth agar and shaking was done at 150rmp for 48 hours at room temperature. The broth culture was filtered by Whatman No.1 filter paper and centrifuged at 6000rmp for 12min. To obtain cell free culture, liquid was re-filtered by Millipore membrane filter (0.2µ). Twenty ml cell free culture was mixed in eighty ml sterilized PDA media by using food poison technique. After the solidification of media, 6mm mycelial plugs of *P. megasperma* were placed at center of PDA petri plates aseptically. PDA petri plates without culture filtrate were served as control. All petri plates were sealed with parafilm and incubated at 25±1°C.

For Trichoderma

To evaluate/check the antifungal activity of Extract of metabolites (culture filtrates) of three *Trichoderma* isolates against *P. megasperma*, technique described by Jeyaseelan *et al.*, (2012) was used with minor modification. Five days old, mycelial plugs of *Trichoderma* isolates were inoculated in 100ml liquid potato dextrose broth (PDB) and shaking was done at 200rpm for 70 hours at room temperature. Then broth culture was centrifuged at 8000rpm for 25min. To obtain cell free culture, broth culture was first filtered by filter paper (Whatman No. 1) and then passed through Millipore membrane (0.34µm). Twenty ml cell free culture was mixed in eighty ml sterilized PDA media by using food poison technique. After the solidification of media, 6mm mycelial plugs of *P. megasperma*were placed at center of PDA petri plates aseptically. PDA petri plates mixed with distilled water instead of culture filtrate served as control. All petri plates were sealed with parafilm and incubated at 25±1°C.

Statistical analysis

The data was analyzed by using factorial test on Statistix software for the interpretations of results. Differences between means were calculated by using LSD test. Percentage inhibition was determined by using following formula (Vincent, 1927). Inhibition percentage (%) = C-T/C × 100

Where C = growth of pathogen in control plate, and T = mycelial growth of pathogen in test plate.

Results

Dual Culture Assay

All the tested biocontrol agents inhibited the mycelial growth of *P. megasperma* and *T. asperellum* showed maximum growth inhibition (67.43%) followed by *T. harzianum* (62.85%) while minimum inhibition was shown by isolate of *T. viride* which was about 46.13% (Table 1).

Table 1. Effect of different biocontrol agents on percent growth inhibition of *Phytophthora megasperma* at different time intervals by dual culture technique.

	Dual culture assay		
Treatments	3rd day	5th day	8th day
T. harzianum	21.07b	43.64a	62.85b
T. aperellum	23.63a	45.24a	67.43a
T. viride	14.42d	31.12c	46.13d
B. subtilis	17.14c	37.94b	51.07c

Means sharing similar letter with in a column are statistically non-significant (P>0.05).

Metabolites

Metabolites of tested biocontrol agent's antagonized the growth of *P. megasperma* under laboratory conditions. Nonvolatile metabolites showed higher growth inhibition of tested pathogen as compared to volatile metabolites. In case of nonvolatile metabolites, *T. asperellum* showed higher percentage

(47.60%) of pathogen mycelial growth inhibition while minimum inhibition was shown by *T. viride* (26.90%). While in case of volatile metabolites, *T. harzianum* showed maximum growth inhibition (38.20%) and minimum inhibition percentage was showed by *T. viride* (13.40%) as compared to all other treatments (Table 2).

Table 2. Effect of metabolites on percent inhibition growth of *Phytophthora megasperma* at different time intervals.

	Nonvolatile metabolites			Volatile metabolites		
Treatment	3rd day	5th day	8th day	3rd day	5th day	8th day
T. harzianum	14.30a	24.50b	41.21b	11.36a	23.43a	38.20a
T. aperellum	16.10a	28.46a	47.60a	9.66a	20.46b	34.13b
T. viride	8.32b	15.30d	26.90d	4.10b	8.80c	13.40d
B. subtilis	9.41b	17.10c	32.44c	5.56b	10.60c	18.36c

Means sharing similar letter with in a column are statistically non-significant (P>0.05).

Discussion

Management of phytopathogens through antagonistic biocontrol agents is more efficient and long lasting approach that is widely adopted in the world. Present study assessed the antifungal ability of different biocontrol agents by dual culture and volatile and nonvolatile metabolites. *T. asperellum* showed highest inhibition among all other tested antagonists in different assays. *T. viride* and *Bacillus subtilis* isolates gave less inhibition percentage of radial growth of tested pathogen as compared to other *Trichoderma* isolates. This may be due to microbial interaction like compatibility, antibiosis and stimulation levels of *Trichoderma* and *Bacillus* against *P. megasperma*. This has also been reported by many researchers (Yuan *et al*., 2012; Raza *et al*., 2013). Antifungal activity of *Bacillus* was also reported by Filippi *et al*., (1989).

Previously similar results about the antagonism of *Trichoderma* isolates against *Phytophthora* spp.were reported by Akgul and Mirik (2008); Osorio-Hernandez *et al*., (2011). Lelay *et al*., (2007) reported the antifungal ability of six *Trichoderma* isolates that inhibited the mycelial growth of *Rosellinia necatrix* by about 14 to 27%. It was attributed to the production of metabolites like viridine, glyotoxins, furanone, trichodermine, and 6-pentyl-α-pyrone.

In present study, *B. subtilis*showed the inhibitory effect against tested pathogen as compared to control treatment. Many researchers reported the production of antifungal compounds of *Bacillus* isolates like gramicidin S, polymyxin, bacitracin, bacilysin, mycobacillin, tyrotricidin, mycosubtilin, iturin, subsporin and fungistatin (Collins and Jacobsen, 2002).

Conclusion

According to present study it is concluded that alternative strategies like biocontrol agents have great potential to manage the growth of phytopathogens. These can be used as component of integrated disease management to obtain organic crops and manage the plant diseases safely as compared to synthetic chemicals that are harmful to human health and environment.

Conflict of interest

"The author(s) declare(s) that there is no conflict of interests regarding the publication of this article".

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