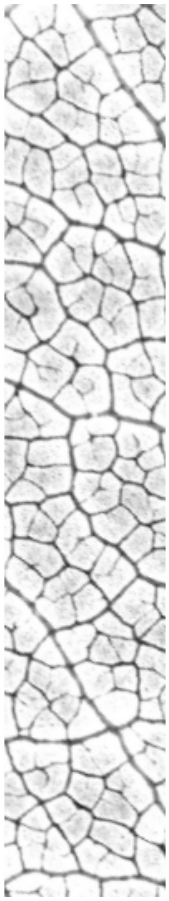

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Antifungal activity of bacteria against the phytopathogens of papaya (*Carica papaya* L.)

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Abstract
The present study was undertaken to evaluate *in vitro* antifungal activity of six bacterial strains procured from MTCC, India against two of the plant pathogenic fungi, *Colletotrichum gloeosporioides* and *Curvularia carica papayae*, affecting papaya. Initial screening was done by dual culture assay and two relatively potent bacteria viz., *Pseudomonas aeruginosa* MTCC 7904 and *Pseudomonas monteilii* MTCC 9796 were selected and further assayed against the pathogens using crude extract of their culture filtrates by poisoned food technique. Both the bacteria exhibited significant antifungal activity against the phytopathogens (*Colletotrichum gloeosporioides* and *Curvularia carica papayae*) screened by complete inhibition of mycelial growth. The results suggest the possible applicability of these two bacteria for the eco-friendly management of papaya phytopathogens after further studies.

Keywords: papaya, phytopathogenic fungi, antifungal bacteria, biocontrol

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INTRODUCTION

Carica papaya L., belonging to family Caricaceae is commonly known as papaya in English, *Papita* in Hindi, *Erandakarkati* in Sanskrit and *Pappayi* in Kannada. Papaya is a powerhouse of nutrients and is available throughout the year. It is a rich source of three powerful antioxidant vitamins (C, A and E); the minerals (magnesium and potassium), the B vitamin, pantothenic acid, foliate and fibers (Milind and Gurditta 2011). Traditionally leaves have been used for treatment of a wide range of ailments like in treatment of malaria, dengue, and jaundice; and in immunomodulatory and antiviral activities. Papaya leaf juice helps to increase white blood cells and platelets, normalizes clotting and repairs the liver (Arvind et al. 2013). Both leaf and fruit of the *C. papaya* possess carotenoids namely β-carotene, lycopene, anthraquinones, glycoside and hence possess medicinal properties like anti-inflammatory, hypoglycaemic, anti-fertility, abortifacient, hepatoprotective and wound healing; recently its antihypertensive and antitumor activities have also been established (Arvind et al. 2013).

Like other plants papaya is also infected by many plant pathogens, which hampers the commercial production of papaya worldwide. Papaya anthracnose caused by the fungus *Colletotrichum gloeosporioides* (Dickman and Alvarez 1983) is one of the most widespread and devastating disease of papaya, especially during storage. It is a major constraint to papaya production as well as to export of the fruit to bigger overseas markets (Ademe et al. 2014). Another important disease affecting papaya is the leaf spot disease caused by *Curvularia carica papayae* (Srivastava and Bilgrami 1963).

Synthetic fungicides are used as the primary means for controlling plant pathogens (Girish et al. 2009a). The papaya diseases are also generally managed by the application of synthetic fungicides. However, excessive usage of synthetic fungicides has many problems associated such as development of resistance, high levels of toxic residues and also there are negative public perceptions about the use of synthetic chemicals as they can cause adverse effects on health and environment (Ademe et al. 2013). This has paved way for the search of alternate eco-friendly methods wherein biocontrol has emerged as a promising alternative. The term biological control applies to the use of microbial antagonists to suppress diseases. The organisms that suppress the pathogens are referred to as the biological control agents (BCA). More broadly, the term biological control also has been applied to the use of natural products obtained from various sources against the phytopathogens (Pal and Gardner 2006). Bacteria have been employed effectively for the control of many plant pathogens (Gopalkrishnan et al. 2010, Tariq et al. 2010, Devakota et al. 2011). Owing to these aspects the present studies were conducted to develop an eco-friendly biocontrol management strategy against

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phytopathogens of papaya *viz*., *Colletotrichum gloeosporioides* and *Curvularia carica papayae* using antifungal bacteria.

MATERIALS AND METHODS

Isolation of *Colletotrichum gloeosporioides* and *Curvularia carica papayae* from Infected Papaya Explants

The pathogens were isolated following the tissue isolation procedure (Dhingra and Sinclair 1995). Anthracnose diseased papaya fruits and the leaf spot infected papaya leaves were collected from different regions of Mandya, Karnataka, India. The diseased part was cut into 1x1 cm segments using sterile blades. The leaf segments were washed thoroughly with running tap water, surface sterilized using sodium hypochlorite solution and washed five times with distilled water. Surface sterilization was not done for the infected fruit segments. The segments were placed aseptically in Petri dish containing Potato Dextrose agar (PDA, Himedia) amended with 100 ppm of chloramphenicol (20 ml / plate). Inoculated plates were incubated at room temperature (RT) with 12 h photo period for 10 days and observed for the growth of the pathogens from the segments. The incubation was continued for 15 days to allow sporulation. The spores were identified microscopically and the presence of *Colletotrichum gloeosporioides* and *Curvularia carica papayae* was confirmed.

Both the isolates were sub-cultured on to the fresh PDA plates by transferring 5.0 mm mycelial agar disc drawn from margin of mycelial mat of *C. gloeosporioides* and *C. carica papayae* and incubated at RT with 12 h photo period for 10 days. Three replicates of each isolates were maintained.

Antagonistic Bacteria

Six bacterial cultures were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India such as *Bacillus amyloliquefaciens* MTTC 10439, *Bacillus cereus* MTTC 9017, *Erwinia* sp. MTTC 2760, *Pseudomonas aeruginosa* MTCC 7904, *Pseudomonas marginalis* MTTC 2758, *Pseudomonas monteilii* MTCC 9796, and were used to carry out further experiments. All the bacterial strains employed in this study are reported to be antagonists of plant pathogens and non-pathogenic to humans (Catalog, MTCC).

All bacterial cultures, except *Pseudomonas* spp., were first streaked on nutrient agar (NA, Himedia) in Petri dishes and a single cell colony was isolated from each culture. A single cell colony of each bacterium was grown on NA slant and was maintained at 4°C. *Pseudomanas* spp., were isolated and maintained on King's B medium (Girish et al. 2009b).

Screening of Bacteria for Antifungal Activity by Dual Culture

The fungi *C. gloeosporioides* and *C. carica papayae* were inoculated (5.0 mm discs) at the center of the solidified PDA medium taken in a 90 mm Petriplate. Each bacterial culture was streaked above and below the fungal disc with sterilized inoculation loop at a distance of 2.0 cm. PDA plates inoculated only with fungi served as control. The Petriplates were then incubated at 37°C for 7-10 days. After incubation the plates were observed for biocontrol of fungi by bacteria (by the formation of zone of inhibition or reduction in the mycelial growth in comparison to control plate). The strains that showed better inhibition of fungal growth were then used for further studies.

Isolation of Ethyl Acetate Fraction from Bacterial Culture Filtrates

The extraction of antifungal ethyl acetate fraction from bacterial culture filtrate was carried out as described by Girish et al. (2009b). 100 ml of nutrient broth (Himedia) taken in 500 ml Erlenmeyer flask was inoculated with a loop of 24h-old-culture of *Pseudomonas aeruginosa* MTCC 7904 and *Pseudomonas monteilii* MTCC 9796 bacteria separately. Totally 1.0 l of medium was inoculated for each bacterium. All the flasks were incubated at 37°C for 72 h. Then the cells were harvested by centrifugation (9000 x g for 10 min at 4°C). The supernatant was collected, the volume of each filtrate was made up to 1.0 l with sterile distilled water, filter sterilized using 0.45 µm membrane filter and stored at 4°C. For extraction, the bacterial culture filtrates were concentrated to 10% of their original volume by using a flask evaporator at 50°C and pH of culture filtrates (100 ml) was adjusted to 3.6 using 1.0 N HCl. Then the culture filtrates were extracted three times with equal volume of ethyl acetate. The aqueous fraction was discarded and the organic extracts of culture filtrates were pooled and evaporated at RT to obtain brownish, semi-solid crude extract.

Preparation of Stock and Control Solutions

Stock solutions (1000 ppm) of each microbial ethyl acetate fraction were prepared by dissolving fractionated material in sterile distilled water containing 0.1% Tween-20 (1.0 mg/ml). Sterilized distilled water containing 0.1% Tween-20 served as control solution (Girish et al. 2009b).

Effect of Ethyl Acetate Fractions of Bacterial Culture Filtrates on Mycelial Growth of *Colletotrichum gloeosporioides* and *Curvularia carica papayae*

Ethyl acetate fraction of *P. aeruginosa* and *P. monteilii* culture filtrates were tested against isolated phytopathogens using poisoned food technique (Girish et al. 2009b). Stock solutions of all ethyl acetate fractions were added at different concentrations separately to sterile PDA to obtain concentrations of 20, 40, 60, 80

Fig. 1. Anthracnose infected papaya fruit

Fig. 2. Leaf spot infected papaya leaf

Fig. 3. Pure culture of *Colletotrichum gloeosporioides* on PDA (10-days-old)

Fig. 4. Pure culture of *Curvularia carica papayae* on PDA (10-days-old)

and 100 ppm. PDA amended with the control solution served as control. About 20 ml of all treated and untreated PDA were poured into separate Petri dishes (9.0 mm diameter), allowed to solidify and inoculated with the five mm mycelial-agar-disc taken from the margin of mycelial mat of seven-day-old culture of *C. gloeosporioides* and *C. carica papayae.* The inoculated Petri dishes were incubated at RT with 12h photoperiod for 10 days. All treatments had three replications and the experiment was repeated two times. After incubation the mycelial growth was recorded in mm, mean colony diameter was determined and the percentage inhibition of mycelial growth was calculated. The colony diameter was compared with the control to measure fungi toxicity. Percent inhibition (PI) with respect to the control was computed using formula PI = (C-T / C) X 100 where C is the colony diameter of the control and T is that of the treated combinations.

RESULTS

Isolation of Phytopathogenic Fungi *Colletotrichum gloeosporioides* and *Curvularia carica papayae* from Infected Papaya Plant Explants

C. gloeosporioides and *C. carica papayae* were isolated from infected fruit and leaves of papaya (**Figs. 1**-**4**). The spores were identified microscopically and the presence of both the fungi was confirmed.

Screening of Bacteria for Antifungal Activity by dual Culture Method

The six different bacterial cultures screened such as *B. amyloliquifaciens* (MTCC 10439), *B. cereus* (MTCC 9017), *Erwinia* sp. (MTCC 2760), *P. aeruginosa* (MTCC 7904), *P. monteilii* (MTCC 9796) and *P. marginalis* (MTCC 2758), effectively suppressed the growth of phytopathogenic fungi *C. gloeosporioides* and *C. carica papayae* showing the zone of inhibition / reduced mycelial growth in comparison to control after 10 days of incubation by dual culture method. *P. aeruginosa* (MTCC 7904) and *P. monteilii* (MTCC 9796) exhibited comparatively better antifungal activity against both the fungi studied (**Fig. 5**; **Table 1**) and were selected for further studies.

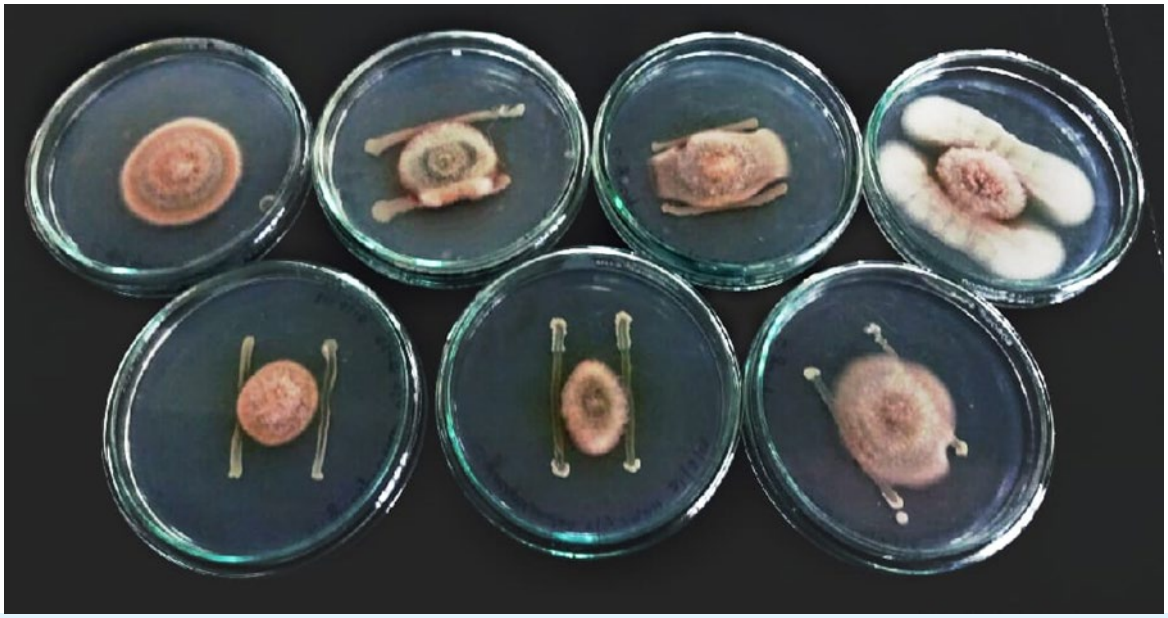

Fig. 5. Antifungal activity of bacteria against *Colletotrichum gloeosporioides* (From left: Control, *Bacillus amyloliquifaciens* MTCC 10439, *Bacillus cereus* MTCC 9017, *Pseudomonas marginalis* MTCC 2758 – upper row; *Pseudomonas aeruginosa* MTCC 7904, *Pseudomonas monteilii* MTCC 9796, *Erwinia* sp. MTCC 2760 – lower row). Similar activity was observed by these bacteria against *Curvularia carica papayae*

Table 1. Antifungal activity of bacteria against *Colletotrichum gloeosporioides* and *Curvularia carica papayae* in dual culture assay

Sl. No.	Bacteria	Antifungal activity against	
		Colletotrichum gloeosporioides	***Curvularia carica papayae***
1.	*Bacillus amyloliquifaciens* MTCC 10439	+	++
2.	*Bacillus cereus* MTCC 9017	++	++
3.	*Pseudomonas marginalis* MTCC 2758	++	++
4.	*Pseudomonas aeruginosa* MTCC 7904	+++	+++
5.	*Pseudomonas monteilii* MTCC 9796	+++	+++
6.	*Erwinia* sp. MTCC 2760	+	++

(+ : inhibition of fungal growth; based on the zone of inhibition / reduced mycelial growth in comparison to control after 10 days of incubation)

Table 2. Amount of extract obtained by the selected antifungal bacteria

Bacteria	Ethyl acetate fraction
Pseudomonas aeruginosa MTCC 7904	1.12 g
Pseudomonas monteilii MTCC 9796	1.03 g

Isolation of Ethyl Acetate Fractions from Bacterial Culture Filtrates

The amount of ethyl acetate fractions obtained from the culture filtrates of two antifungal bacteria are given in **Table 2**.

Effect of Ethyl Acetate Fractions of *Pseudomonas aeruginosa* MTCC 7904 and *Pseudomonas monteilii* MTCC 9796 Culture Filtrates on *Colletotrichum gloeosporioides* and *Curvularia carica papayae*

Ethyl acetate fraction of *P. aeruginosa* MTCC 7904 culture filtrate exhibited significant antifungal activity by resulting in complete suppression of mycelial growth of *C. gloeosporioides* at 100 ppm concentration, and of *C. carica papayae* at 80 ppm concentration (**Figs. 6** & **7**; **Table 3**). *P. monteilii* MTCC 9796 ethyl acetate fraction also exhibited similar antifungal activity by completely suppressing the mycelial growth of *C. gloeosporioides* at 100 ppm concentration, and of *C. carica papayae* at 80 ppm concentration (**Figs. 8** & **9**; **Table 4**).

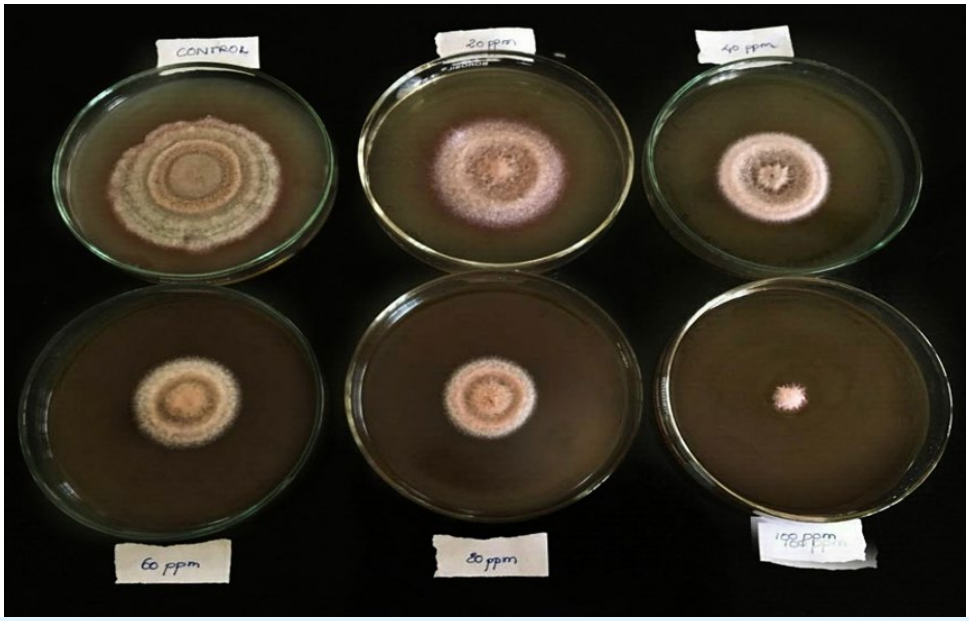

Fig. 6. Effect of ethyl acetate fraction of *Pseudomonas aeruginosa* MTCC 7904 at concentrations of 20, 40, 60, 80 and 100 ppm respectively against *Colletotrichum gloeosporioides*

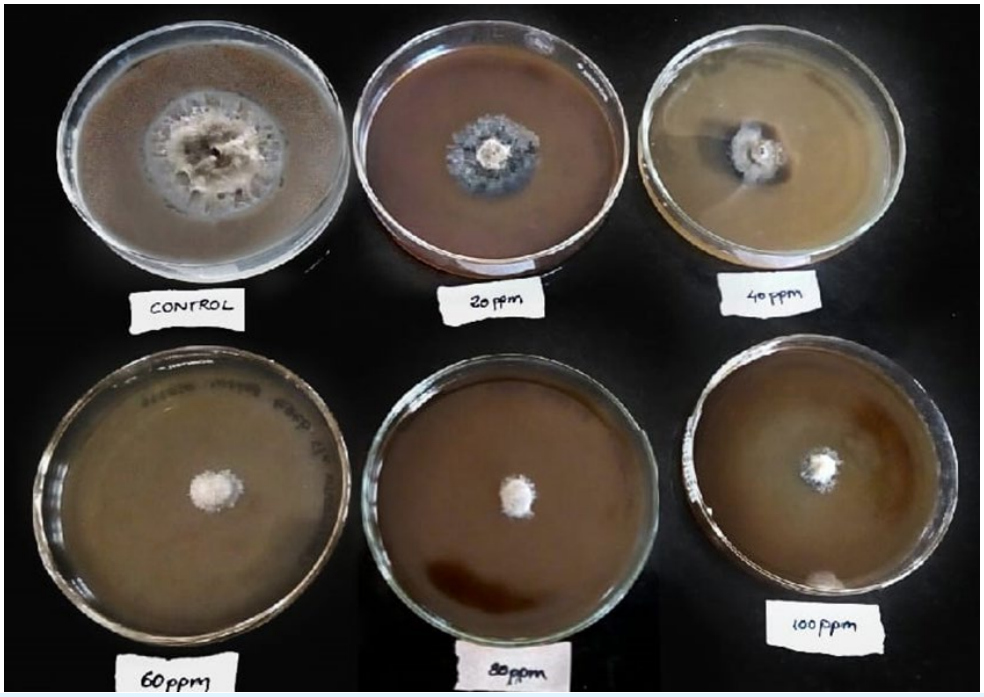

Fig. 7. Effect of ethyl acetate fraction of *Pseudomonas aeruginosa* MTCC 7904 at concentrations of 20, 40, 60, 80 and 100 ppm respectively against *Curvularia carica papayae*

Table 3. Effect of ethyl acetate fraction of *Pseudomonas aeruginosa* MTCC 7904 at different concentrations against *Colletotrichum gloeosporioides* and *Curvularia carica papayae*

Concentrations (Ethyl acetate extract of culture filtrate)	***Colletotrichum gloeosporioides***		***Curvularia carica papayae***	
	Mycelial growth (mm)	**Growth inhibition (%)**	**Mycelial growth (mm)**	**Growth inhibition (%)**
0 ppm (Control)	73.5 ± 0.89	0.0 ± 0.0	49.0 ± 1.21	0.0 ± 0.0
20 ppm	41.3 ± 0.33	42.9 ± 0.67	22.5 ± 0.57	53.93 ± 1.00
40 ppm	35.6 ± 0.57	51.0 ± 1.00	19.6 ± 0.74	59.9 ± 1.33
60 ppm	30.6 ± 0.67	57.8 ± 1.17	16.8 ± 0.67	65.6 ± 1.21
80 ppm	27.5 ± 0.57	61.9 ± 0.89	0.0 ± 0.0	100 ± 0.0
100 ppm	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0	100 ± 0.0

The values are means of three replicates ± standard error

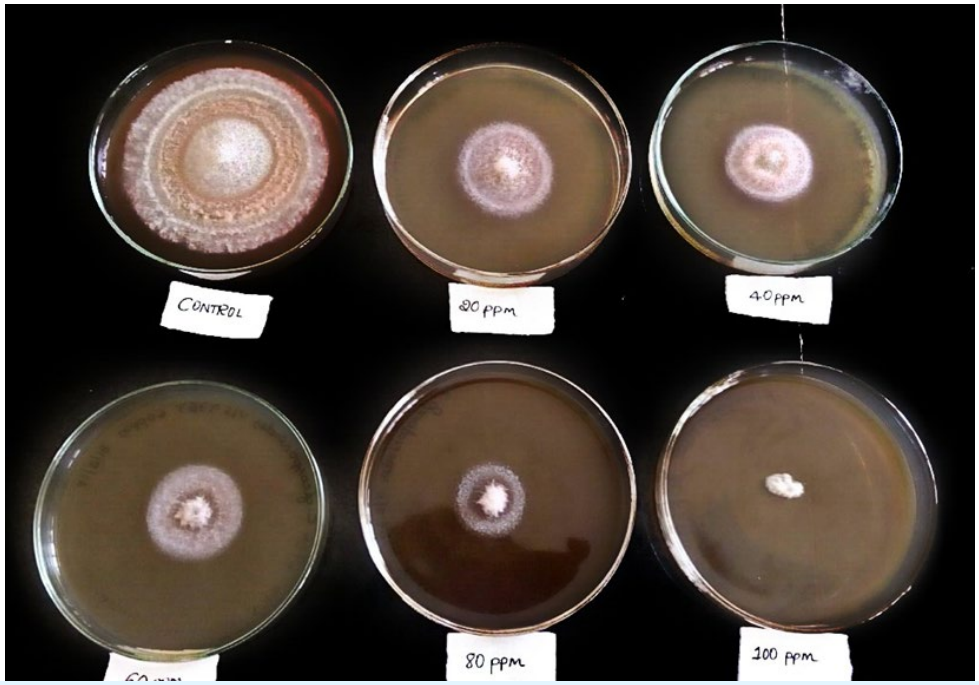

Fig. 8. Effect of Ethyl acetate fraction of *Pseudomonas monteilii* MTCC 9796 at concentrations of 20, 40, 60, 80 and 100 ppm respectively against *Colletotrichum gloeosporioides*

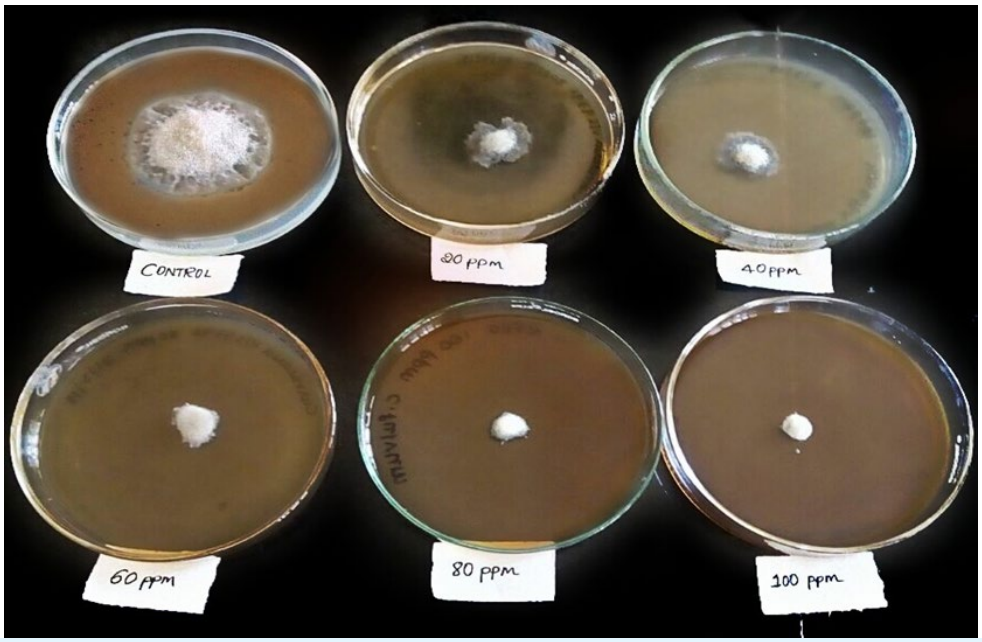

Fig. 9. Effect of ethyl acetate fraction of *Pseudomonas monteilii* MTCC 9796 at concentrations of 20, 40, 60, 80 and 100 ppm respectively against *Curvularia carica papayae*

Table 4. Effect of ethyl acetate fraction of *Pseudomonas monteilii* MTCC 9796 at different concentrations against *Colletotrichum gloeosporioides* and *Curvularia carica papayae*

Concentrations (Ethyl acetate extract of culture filtrate)	*Colletotrichum gloeosporioides*		*Curvularia carica papayae*	
	Mycelial growth (mm)	Growth inhibition (%)	Mycelial growth (mm)	Growth inhibition (%)
0 ppm (Control)	74.6 ± 1.21	0.0 ± 0.0	48.0 ± 1.33	0.0 ± 0.0
20 ppm	45.6 ± 0.89	34.9 ± 1.55	18.5 ± 0.33	47.8 ± 1.0
40 ppm	37.5 ± 0.57	49.74 ± 1.17	17.1 ± 0.74	60.46 ± 1.45
60 ppm	27.8 ± 0.33	62.7 ± 1.0	12.0 ± 0.67	74.36 ± 1.17
80 ppm	24.5 ± 0.67	67.16 ± 1.21	0.0 ± 0.0	100 ± 0.0
100 pppm	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0	100 ± 0.0

The values are means of three replicates ± standard error

DISCUSSION

The cultivation of papaya (*Carica papaya* L.) has great potential and its demand in national and international market is ever increasing owing to its substantial medicinal properties. However, the papaya crop is being infected by viral, fungal, bacterial, nematodal and phytoplasma diseases (Nishijima 1994). *Colletotrichum gloeosporioides* which cause anthracnose disease in papaya and *Curvularia carica papayae* causing leaf spot disease in papaya are major pathogens. These diseases can be controlled by spraying chemicals. However, the synthetic chemicals, in general, lead to the emergence of resistant pathogens, cause damage to the environment and adversely affect human health due to toxic residues (Vurro and Gressel 2006). Thus the use of eco-friendly alternative approaches for the management of plant diseases is very much essential. Biological control of plant diseases using microorganisms provides a possible alternative for the effective management of plant diseases by decreasing the input of agrochemicals (Lugtenberg and Bloemberg 2004, Babbal et al. 2017).

In the present study the bacteria procured from MTCC such as *Bacillus amyloliquefaciens* MTCC 10439, *Bacillus cereus* MTCC 9017, *Erwinia* sp. MTCC 2760, *Pseudomonas aeruginosa* MTCC 7904, *Pseudomonas marginalis* MTCC 2758 and *Pseudomonas monteilii* MTCC 9796, were screened by dual culture method to select bacteria having the antagonistic properties against *C. gloeosporioides* and *C. carica papayae* and then by evaluation of ethyl acetate fraction of culture filtrate of the selected bacteria against the phytopathogens by food poisoning method. Dual culturing and food poisoning are the methods regularly employed by researchers for screening antifungal activity of bacteria against the phytopathogens (Farah and Nasreen 2016, Hammami et al. 2013, Pankaj Kumar et al. 2012, Yang et al. 2017). Both of the selected bacteria effectively suppressed the growth of *C. gloeosporioides* and *C. carica papayae*. Ethyl acetate fractions of both *P. aeruginosa* MTCC 7904 and *P. monteilii* MTCC 9796 showed complete growth inhibition of *C. gloeosporioides* at the concentration of 100 ppm and complete growth inhibition of *C. carica papayae* at the concentration of 80 ppm. With the increase in the concentration of bacterial extracts a significant decrease in the mycelial growth of the pathogens was observed. This may be due to the exposure of pathogen to increasing concentrations of antifungal secondary metabolites produced by the bacteria (Girish et al. 2009b).

These results are in accordance to many previous reports. *Pseudomonas* strains isolated from tomato and pepper plants rhizosphere soil, significantly inhibited *Alternaria alternata, Sclerotinia sclerotiorum* and *Fusarium solani* in dual culture antagonism assay (Hammami et al. 2013). *P. aeruginosa* and *Bacillus subtilis* isolated from rhizosphere significantly inhibited *Fusarium oxysporum* and *Rhizoctonia solani* causing soft cottony leak of cucurbitaceous fruits when tested using dual culture method (Farah and Nasreen 2016). Cell-free culture filtrate of *Bacillus* sp. BPR7 caused colony growth inhibition of all the test pathogens such as *Macrophomina phaseolina, Fusarium oxysporum, F. solani, S. sclerotiorum, R. solani* and *Colletotrichum* sp., *in vitro* (Pankaj Kumar et al. 2012). Crude extract from *Streptomyces cochorusii* strain NF0919 culture filtrate provided better disease control than synthetic fungicides against the rice sheath blight (RSB) pathogen *R. solani* (Yang et al. 2017).

CONCLUSION

The present study provides a basis for the development of effective and eco-friendly biocontrol management strategy against *C. gloeosporioides* and *C. carica papayae*. The high antifungal efficacies of the fractions from the *P. aeruginosa* MTCC 7904 and *P. monteilii* MTCC 9796 bacteria at low concentrations indicates a possibility of their use as safe alternative to chemical fungicides for effective management of anthracnose and leaf spot disease of papaya. However, further studies such as preparation of bioformulations, green house and field studies are required to effectively employ these bacteria against *C. gloeosporioides* and *C. carica papayae* during cultivation. It will be advantageous, if this method can be developed as bio-pesticides and also as an integrated disease management strategy.

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