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Evaluation of antagonistic bacteria for the biocontrol of *Phomopsis azadirachtae,* the die-back of neem pathogen

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Abstract

In the current investigation, antagonistic bacteria procured from MTCC, IMTECH, Chandigarh were evaluated for antifungal activity against *Phomopsis azadirachtae*, the fungus causing die-back of neem by dual culture method. Five bacteria viz., *Bacillus amyloliquefaciens, B. cereus, B. subtilis, Erwinia* sp. and *Pseudomonas aeruginosa* exhibited excellent inhibition of the pathogen. Then the ethyl acetate extracts of culture filtrates of these bacteria were screened against *P. azadirachtae* by poisoned-food method at different concentrations such as 5, 10, 15, 20, 25 and 30 ppm. All the tested bacterial strains completely inhibited *P. azadirachtae* growth at 20 ppm concentration. These bacteria could be employed for the biocontrol as well as for the integrated management of *P. azadirachtae*.

Key words : *Phomopsis azadirachtae*, die-back, Neem, antagonistic bacteria, biocontrol.

Neem (*Azadirahcta indica* A. Juss.) is one of the most valuable medicinal trees with an array of therapeutic properties[10,14]. Neem products have been found to have antiallergic, antidermatic, anti-inflammatory, insecticidal, larvicidal, nematicidal, spermicidal, pesticidal properties as well as antimicrobial activities including antifungal activity[10,15]. However, neem in spite of all its medicinal attributes is also vulnerable to microbial diseases and several bacteria and fungi cause diseases on neem[9].

The most destructive neem pathogen currently is a fungus, *Phomopsis azadirachtae* Sateesh, Bhat and Devaki causing die-back[16]. The systemic fungicides like bavistin is reported to control the pathogen[11], but synthetic fungicides have residual issues, cause pollution of soil or water negatively affecting the associated microbiota[5,17]. Therefore, it is essential to have an alternative approach that is ecofriendly to manage this pathogen.

Biocontrol employing microorganisms

has become an effective alternative method to manage plant pathogens that helps to reduce the input of agrochemicals[3,4]. Microbes especially antagonistic bacteria with their varied biocontrol mechanisms including production of lytic enzymes, siderophores, and antibiotics efficiently suppress the growth of plant pathogens[6,13]. Bacterial cultures procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India have been reported to have antifungal activity against phytopathogenic fungi[8,12].

In this present study, antagonistic bacteria (MTTC strains) were screened for the *in vitro* biocontrol of *P. azadirachtae* initially by dual culture method and then by employing ethyl acetate fraction of culture filtrates of tested bacteria. Screening of antagonistic bacteria (*Bacillus cereus, B. subtilis,* and *Pseudomonas aeruginosa*) for antifungal activity against *P. azadirachtae* is reported[12]. However, the strains used in that study were different from the strains employed in the present study. Also, this is the first report of evaluating *B. amyloliquefaciens* and *Erwinia* sp., against *P. azadirachtae.*

Antagonistic bacteria :

Ten bacterial cultures were procured from MTCC, IMTECH, Chandigarh, India such as *Bacillus amyloliquefaciens* MTTC 10439, *Bacillus cereus* MTTC 9017, *B. subtilis* MTCC 8142, *Enterobacter aerogenes* MTCC 8558, *Erwinia* sp. MTTC 2760, *Pseudomonas aeruginosa* MTCC 7904, *Pseudomonas fluorescens* MTCC 9768, *Pseudomonas lurida* MTCC 9767, *Pseudomonas marginalis* MTTC 2758, *Pseudomonas monteilii* MTCC 9796. 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 streaked on nutrient agar (NA, Himedia) plates and a single cell colony was isolated from each culture, which was grown on NA slant and maintained at 4°C. Similarly *Pseudomanas* spp., were isolated and maintained on King's B medium[8].

Screening of bacteria against Phomopsis azadirachtae *by dual-culture method :*

5.0 mm mycelial disc of 7-day-old *P. azadirachtae* culture was inoculated at the centre of Potato Dextrose Agar (PDA, Himedia) plate and bacterial inoculum was streaked as a circle around fungal disc. All 10 bacterial isolates were subjected for similar procedure on individual PDA plates. Control PDA plates had only the fungal disc at the centre. All the plates, after inoculation, were maintained for 7-10 days at 37°C. After incubation, plates were examined for antifungal activity (reduced mycelial growth of pathogen in test plates than the control plate). Bacterial strains exhibiting better suppression of fungal growth were chosen for further evaluation.

Isolation of ethyl acetate fraction from bacterial culture filtrate :

Procedure reported by Girish *et al.*[12] has been employed to extract antifungal ethyl acetate fraction from bacterial culture filtrate. 100 ml nutrient broth taken in a 500 ml flask was inoculated with selected bacterium culture. 10 flasks were inoculated in total for each bacterium. All the flasks were maintained for

72 hours at 37°C. Then, the cells were separated employing centrifugation (9000xg for 10 min at 4ºC). The supernatant obtained *i.e*. culture filtrate was filtered through a 0.45 µm membrane filter (Sartorius, Gottingen, Germany) after diluting with sterile distilled water to a volume of 1.5 l. Using a flash evaporator at 50°C culture filtrates were concentrated to 150 ml and with 1.0 N HCl their pH was made to 3.6. Then the culture filtrates were extracted three times employing ethyl acetate at equal volume. While aqueous fraction was discarded, brownish, semi-solid crude extract obtained by evaporating pooled organic extracts of culture filtrates at room temperature (RT) was collected for further work.

Bioassay of antifungal activity of ethyl acetate extract :

Bacterial ethyl acetate fraction stock solution (1000 ppm) was prepared by dissolving the extract in the sterile distilled water having 0.1 percent Tween-20 (1.0 mg/ml). Control solution had sterile distilled water with 0.1 percent Tween-20.[12]

The poisoned-food technique was employed to evaluate ethyl acetate fraction of bacterial culture filtrate against pathogen. Stock solution of ethyl acetate fraction was incorporated separately into sterile PDA to have various concentrations (5, 10, 15, 20, 25 and 30 ppm). PDA plates with 30 ppm of control solution acted as control. About 20 ml of all treated PDA were transferred onto individual 9.0 mm diameter Petri plates. After solidification plates were inoculated with a 5 mm mycelial-agar disc of *P. azadirachtae* culture (7-day-old) and incubated for 10 days at RT with 12 h photoperiod. Experiment was conducted twice having each treatment in triplicate. The amount of ethyl acetate fraction necessary to completely suppress mycelial growth of pathogen was noted down. Average colony diameter was calculated and compared with control plates to estimate fungitoxicity. After 15 days of incubation, the number of pycnidia developed was noted down.

Screening of bacteria against Phomopsis azadirachtae *by dual-culture method :*

Out of ten different bacterial species that were screened, five bacterial strains such as *Bacillus amyloliquefaciens* MTTC 10439, *Bacillus cereus* MTTC 9017, *Bacillus subtilis* MTCC 8142, *Erwinia* sp. MTTC 2760 and *Pseudomonas aeruginosa* MTCC 7904 exhibited good inhibition of *P. azadirachtae* growth in the dual culture method (Figure 1) and were selected.

Effect of ethyl acetate fraction of bacterial culture filtrates on growth of Phomopsis azadirachtae *:*

All the tested bacterial culture filtrates inhibited *P. azadirachtae* growth at 5, 10, 15, 20, 25 and 30 ppm concentrations of their ethyl acetate fraction and at 20 ppm concentration total suppression of mycelial growth as well as pycnidial formation of pathogen was observed (Figure 2; Tables 1 & 2).

The excessive application of chemical pesticides to maintain good health and high productivity of crops has harmful effects on nature including animals and humans[7]. The biological methods of managing plant diseases provide an eco-friendly alternative to have

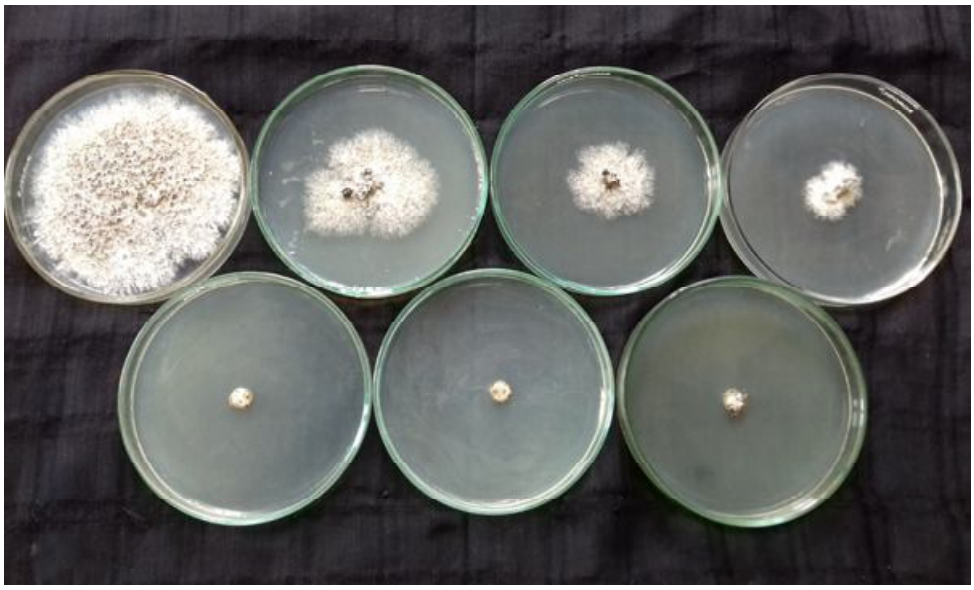

Figure 1. (A) *Bacillus amyloliquefaciens* MTTC 10439; (B) *Bacillus cereus* MTTC 9017; (C) *Bacillus subtilis* MTCC 8142; (D) *Erwinia* sp. MTTC 2760; (E) *Pseudomonas aeruginosa* MTCC 7904 showing good inhibition of *Phomopsis azadirachtae* in dual culture method; (F) Control plate

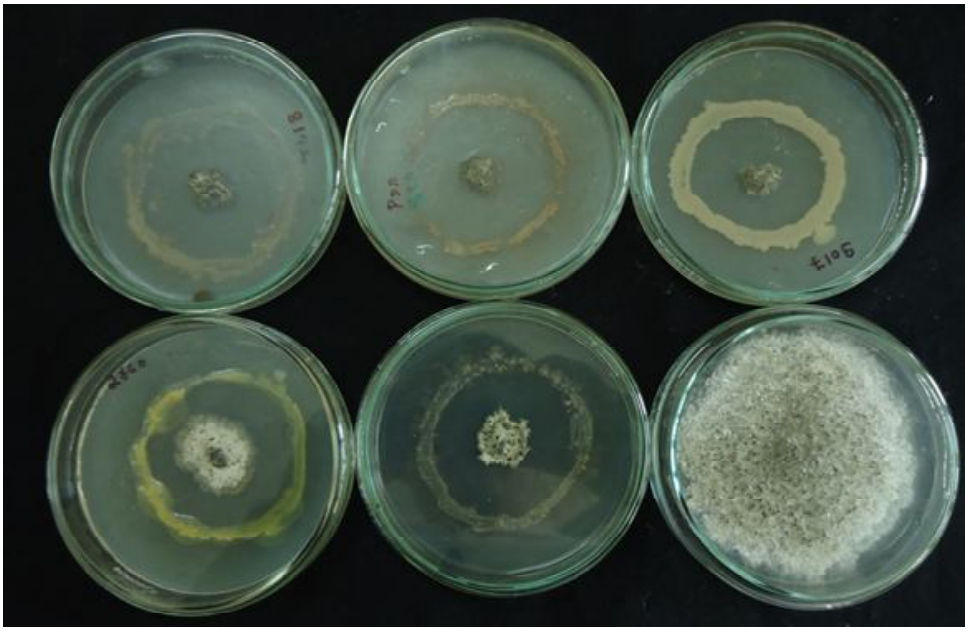

Figure 2. Effect of ethyl acetate fraction of *Bacillus amyloliquefaciens* MTTC 10439 against *Phomopsis azadirachtae* at different concentrations (Similar results were observed with all other four bacterial extracts tested)

Table-1. Effect of ethyl acetate fraction of bacteria at different concentrations on mycelial growth of *Phomopsis azadirachtae*

Concentrations	*Bacillus amyloliquefaciens* MTTC 10439		*Bacillus cereus* MTTC 9017		*Bacillus subtilis* MTCC 8142	
	Diameter of the mycelial mat (mm)	Growth inhibition (%)	Diameter of the mycelial mat (mm)	Growth inhibition (%)	Diameter of the mycelial mat (mm)	Growth inhibition (%)
Control	90.0	0	90.0	0	90.0	0
5 ppm	48.66 ± 1.89	45.9 ± 2.09	45.0 ± 1.52	49.95 ± 1.69	44.33 ± 1.74	50.7 ± 1.93
10 ppm	32.0 ± 0.73	64.4 ± 0.8	26.0 ± 0.73	68.1 ± 2.03	31.66 ± 1.2	64.7 ± 1.32
15 ppm	22.83 ± 1.04	74.58 ± 1.15	17.66 ± 1.2	80.33 ± 1.34	15.33 ± 1.33	82.9 ± 1.47
20 ppm	0	100	0	100	0	100
25 ppm	0	100	0	100	0	100
30 ppm	0	100	0	100	0	100
Concentrations	*Erwinia* sp. MTTC 2760		*Pseudomonas aeruginosa* MTCC 7904			
	Diameter of the mycelial mat (mm)	% growth inhibition	Diameter of the mycelial mat (mm)	% growth inhibition		
Control	90.0	0	90.0	0		
5 ppm	45.5 ± 1.31	49.4 ± 1.46	44.0 ± 1.54	51.05 ± 1.72		
10 ppm	36.33 ± 1.49	59.58 ± 1.66	35.0 ± 1.34	61.06 ± 1.49		
15 ppm	28.66 ± 0.84	68.08 ± 0.93	16.5 ± 0.76	81.63 ± 0.85		
20 ppm	0	100	0	100		
25 ppm	0	100	0	100		
30 ppm	0	100	0	100		

Values are the average of two experiments, each with three replicates ± SE

sustainable agriculture. Microbial antagonists with their unique mechanisms, both direct and indirect, effectively inhibit fungal pathogens causing plant diseases[20]. Among microbes antagonistic bacteria suppressing plant pathogenic fungi have received much attention[1]. Many bacterial genera have been reported to have immense biocontrol potential against various plant diseases[2].

In the present study, five bacteria out of the ten bacterial isolates obtained from MTCC, IMTECH, Chandigarh *viz., Bacillus amyloliquefaciens* MTTC 10439, *Bacillus*

Table-2. Effect of ethyl acetate fraction of bacteria at different concentrations on pycnidia of *Phomopsis azadirachtae*

Concentrations	Number of pycnidia of *Phomopsis azadirachtae*				
	Bacillus amyloliquefaciens MTTC 10439	*Bacillus cereus* MTTC 9017	*Bacillus subtilis* MTCC 8142	*Erwinia* sp. MTTC 2760	*Pseudomonas aeruginosa* MTCC 7904
Control	213.33 ± 3.98	224.0 ± 3.5	222.66 ± 2.62	228.16 ± 3.68	218.66 ± 3.17
5 ppm	77.0 ± 3.16	73.66 ± 2.04	73.5 ± 2.02	78.33 ± 2.02	72.66 ± 2.12
10 ppm	42.5 ± 3.2	35.33 ± 2.18	44.0 ± 2.01	49.33 ± 2.12	36.16 ± 1.74
15 ppm	13.5 ± 2.04	12.8 ± 1.3	15.83 ± 1.4	20.66 ± 1.47	13.66 ± 1.8
20 ppm	0	0	0	0	0
25 ppm	0	0	0	0	0
30 ppm	0	0	0	0	0

Values are the average of two experiments, each with three replicates ± SE

cereus MTTC 9017, *B. subtilis* MTCC 8142, *Erwinia* sp. MTTC 2760 and *Pseudomonas aeruginosa* MTCC 7904 suppressed the growth of *P. azadirachtae* more efficiently in dual culture technique. In poisoned-food method, at the concentration of 20 ppm, ethyl acetate fractions of all the five selected bacteria completely inhibited *P. azadirachtae* growth. The bacterial extract also showed significant reduction in the mycelial growth as well as pycnidia production. This is in accordance with reports on employing bacterial cultures for the biocontrol of plant pathogens[8,19]. Dual culture and poisoned-food techniques have been regularly employed to study bacteria for antifungal activity.[8,12,20] *Pseudomonas aeruginosa* MTCC 7904 and *Pseudomonas monteilii* MTCC 9796 signifi-cantly inhibited the growth of phytopathogens *Colletotrichum gloeosporioides* and *Curvularia carica papaya*, both in dual culture and poisoned-food assays[8]. Bacterial endophytes were screened against fungal pathogens of tomato such as *Alternaria solani, Botrytis cinerea*, *Fusarium solani*, *Rhizoctonia solani* and *Verticillium lateritium* through dual culture assay and *Bacillus siamensis* strain NKIT9 was found to be highly effective against all the pathogenic fungi tested[19].

Though antagonistic bacteria could be isolated from natural sources, using bacteria already deposited in a culture collection centre has the advantage of reducing a lot of labour and time required to isolate, characterize and precisely identifying them. Culture collections provide the authentic microbial cultures for high quality research. Microbial Type Culture Collection (IMTECH, Chandigarh) is one of the active and reputed culture collections in India[18].

Bacteria are rich sources of numerous natural compounds including secondary metabolites having antagonistic activity against various phytopathogens[2]. The significant antifungal potential observed by the bacterial extracts in this study might be due to the

presence of a few such antagonistic secondary metabolites in the extracts. However, there is a need to better understand the biochemistry of biopesticides derived from bacterial products for effective application of them for the biocontrol of plant pathogens[1]. This warrants the need of further research.

In conclusion, development of eco-friendly strategies for the control of die-back of neem is the need of the hour. In current investigation, *in vitro* evaluation of MTCC bacterial cultures against *P. azadirachtae* has revealed their effective antagonistic potential by complete suppression of the growth of *P. azadirachtae*. Thus, these bacteria could be further studied to develop both a biopesticide as well as an integrated disease control strategy against *P. azadirachtae*.

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