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In vitro Biocontrol of *Phomopsis azadirachtae* by *Andrographis paniculata* Rhizobacteria

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Abstract

The *Phomopsis azadirachtae* is a fungal phytopathogen that incites die-back disease on neem. Management of this disease has become very important presently due to its fast spread. Though chemical control is possible, negative aspects of chemicals on ecosystem is making alternative methods a priority. Use of biopesticides mainly consisting of microorganisms is gaining importance as an alternative for synthetic fungicides. Rhizosphere of medicinal plants is a good source for antagonistic bacteria. In this study bacteria were isolated from rhizosphere of *Andrographis paniculata*, a medicinal plant. They were initially tested for antifungal potential against *P. azadirachtae* using dual culture approach. Three rhizobacteria that substantially inhibited fungal growth were characterized using standard biochemical tests. Later by employing the poison-food method, ethyl acetate extracts of the three chosen bacterial culture filtrate were screened for *P. azadirachtae* growth inhibition. All three rhizobacteria substantially prevented the *P. azadirachtae* growth and could be good candidates for the development of a biocontrol strategy to manage *P. azadirachtae*, but this demands further research.

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Introduction

A member from the Meliaceae family, neem (*Azadirachta indica*) is frequently described as "Indian lilac" or "Margosa." Insecticides, pesticides, and agrochemicals can be found naturally in neem.[1] Since ancient times, Indians have been aware of neem's medicinal benefits. Ayurvedic, homoeopathic, and unani medicine have all made substantial use of neem. Numerous pharmacological properties of neem leaves include anti-hyperglycemia, anti-inflammatory, anti-malaria, anti-ulcer, antiviral, antibacterial, antifungal, antioxidant, anti-mutagenic, anti-carcinogenic and immune modulatory. Neem is an integral part of Indian rural medicine.[1,2]

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Even after having antimicrobial potential, neem is susceptible to the infections and diseases caused by microorganisms. Many bacterial and fungal pathogens incite disease on neem.[3] Die-back disease caused by *Phomopsis azadirachtae* Sateesh, Bhat, and Devaki[3,4] is presently a major harmful disease of neem. The disease results in severe decrease of evergreen canopy and also nearly 100% loss in fruit production is observed. This severely affects the health of the plant as well as seed availability.[5] *P. azadirachtae*, is disseminating at a very fast rate[3] and thus proper management is the need of the hour.

P. azadirachtae is reported to be inhibited by a few chemicals.[6] Since fungicides are known to be biohazardous, alternate methods (biofungicides) for managing plant diseases must be developed.[7] Microbial communities in natural ecosystems, especially in soil, are known to inhibit the growth of pathogenic microbes by their antagonistic properties.[8]

Andrographis paniculata, a medicinal plant and annual herbaceous plant, is a member of the Acanthaceae family. It is extensively grown in China, India, southern Asia, and some regions of Europe. Because of the different pharmacological effects it has, including antimicrobial activities, *A. paniculata* has been used for generations as a medicine.[9] Review of literature indicates that majority of research works are concentrated on antimicrobial activities of *A. paniculata* extracts.[9,10] Work on screening of *A. paniculata* rhizosphere bacteria for antifungal activity is nil. Owing to this the present investigations were carried out with the objectives of isolation of bacteria from *A. paniculata* rhizosphere and evaluation of their antagonistic potential against *P. azadirachtae*.

Materials and Methods

Isolation of Rhizobacteria

Samples of *Andrographis paniculata* rhizosphere soil were gathered. 1.0 g soil sample was subjected to serial dilution using sterile physiological saline up to 10^{-9} dilution. Using a sterile spreader, inoculum of 0.1 ml from 10^{-7}, 10^{-8}, 10^{-9} dilutions was spread on solidified nutrient agar (NA) plates aseptically. Subsequently, the plates were maintained at 37°C over 24 - 48 hours. Following incubation, streaking was used to pure-culture the distinct-looking cultures onto nutrient agar slants, which were then kept at 4°C until needed.

Screening of rhizobacteria by Dual-Culture Method against *Phomopsis azadirachtae*

Sterile PDA (potato dextrose agar) medium was poured (20 ml) to sterile Petriplates and allowed to solidify. 5.0 mm mycelial disc of *P. azadirachtae* culture (7-days-old) was placed in middle of the plate, while bacterium was streak inoculated above the fungal disc at a distance of 2.0 cm. All 10 bacterial isolates underwent equal process. The control group consisted of PDA plates that were just inoculated with the fungal disc. For 7-10 days, inoculated Petriplates were incubated at 30°C. After incubation, plates were examined for bacterial antifungal activity (a decline in fungal mycelial growth and formation of an inhibition zone between bacterial streak and fungal culture). Strains that demonstrated strongest suppression of fungal growth were chosen for additional research.

Characterization of rhizobacteria having Substantial Antagonism against *Phomopsis azadirachtae*

Apart from staining, biochemical assays, including IMViC, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, triple-sugar iron agar, as well as catalase were carried out in compliance with standard procedures[11] to identify the chosen bacterial strains.

Extraction of Ethyl Acetate Fraction from the Filtrates of Bacterial Cultures

100 ml nutrient broth taken in a 500 ml Erlenmeyer flask was inoculated with a loop of rhizobacteria culture. Each bacterium was inoculated into ten flasks. All of the flasks were maintained at 37°C for 72 hours. The cells were then separated and the supernatant was collected using centrifugation (9000xg, 10 min, 4°C). This culture filtrate was filtered using 0.45 µm membrane filter (Sartorius, Gottingen, Germany), and diluted to 1.5 litres adding sterile distilled water. Employing a flash evaporator at 50°C, culture filtrates volume was condensed to 10 per cent of the original volume (i.e. from 1.5 litres to 150 ml). 150 ml of bacterial culture was made to have a pH of 3.6 using 1.0 N HCl in order to be extracted. The culture filtrates, using the same amount of ethyl acetate, were extracted three times, and each time the organic fraction was collected. A brownish, semi-solid crude extract was obtained after pooling as

well as evaporating organic extracts around room temperature. It was then weighed and kept at 4°C.

Screening of Ethyl Acetate Extracts for Antifungal Potential

A bacterial ethyl acetate fraction stock solution (1000 ppm) was made by dissolving the obtained fraction in sterilized distilled water with 1.0 mg/ml of Tween-20 (0.1%). 0.1% Tween-20 solution in sterile distilled water served as control.

The poison-food method was used to screen the antifungal activity of bacterial ethyl acetate fraction. In the initial screening, several concentrations, 2, 4, 6, 8, and 10 ppm, were obtained by adding appropriate portion of ethyl acetate fraction stock solution into 100 ml of sterile PDA medium in flasks, individually. Similarly concentrations of 20, 40, 50, 60, 80 and 100 ppm were prepared in further screening. PDA (100 ml) consisting either 10 ppm or 100 ppm of control solution was used as control. Twenty millilitres from every treated PDA were added onto separate Petri dishes of 9.0 mm diameter, left to set, and then inoculated with a 5.0 mm mycelial-agar disc of *P. azadirachtae* culture (7 days old) at the centre. Inoculated Petri dishes were then maintained at room temperature with 12-hour photoperiod for 10 days. Experiment was performed twice, with three replications each time. After incubation, each ethyl acetate fraction concentration's effect on mycelial development was noted down in terms of colony diameter. To estimate fungitoxicity, the colony diameter in test plates was compared with that of the control plate. Pycnidial number was noted down after 15 days of incubation.

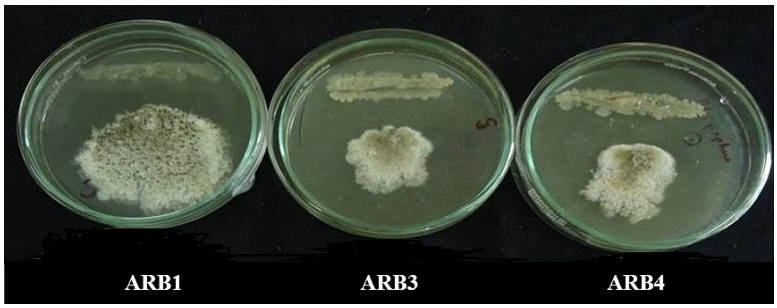

Fig. 1: Inhibition of *Phomopsis azadirachtae* by rhizobacteria in dual culture method

Table 1: Biochemical characterization of rhizosphere bacteria

Tests	ARB1	ARB3	ARB4
Gram Staining	Gram positive	Gram-positive	Gram negative
Shape	cocci	cocci	cocci
Catalase test	Positive	Positive	Negative
Indole	Positive	Positive	Positive
Methyl Red	Positive	Negative	Positive
Citrate utilization	Positive	Negative	Positive
Triple sugar iron agar	AlS, AcB, Gp	AlS, AcB, Gp	AlS, AcB, Gp, H_2S
Voges Proskauer	Negative	Positive	Negative
Starch hydrolysis	Positive	Positive	Positive
Casein hydrolysis	Negative	Negative	Negative
Gelatin hydrolysis	Positive	Positive	Positive

AlS - alkaline slant, AcB - acidic butt and Gp - gas production

Results

Isolation and Selection of rhizobacterial Strains

The rhizosphere soil yielded ten distinct bacterial species. The bacterial species were named as isolates ARB1 to ARB10 (ARB *Andrographis* Rhizobacteria). Three bacterial strains (ARB1, ARB3, and ARB4)

that showed the best inhibition activity (a decline in fungal mycelial growth and formation of an inhibition zone between bacterial streak and fungal culture) in the dual culture method (Fig. 1) were selected.

Biochemical Characterization of rhizobacteria

Table 1 lists the outcomes of the biochemical and staining tests. Based on the results of biochemical tests conducted it was not possible to precisely identify the bacteria using Bergey's manual of determinative bacteriology. Few more biochemical tests and molecular characterization are required to identify the bacteria precisely.

Extraction of Ethyl Acetate Fraction from the Filtrates of Bacterial Cultures

Table 2 illustrates the quantity of ethyl acetate fractions extracted from culture filtrates of three rhizobacteria.

Screening of Ethyl Acetate Extracts for Antifungal Potential

In 2, 4, 6, 8, and 10 ppm concentrations of their ethyl acetate fraction, none of the three rhizobacteria (ARB1, ARB3, and ARB4) could totally prevent *Phomopsis azadirachtae* from growing. As a result, the experiment was conducted again with higher ethyl acetate fraction concentrations (20, 40, 50, 60, 80, and 100 ppm). Around 40 ppm concentration of their ethyl acetate component, all showed total suppression of the fungal mycelial development (Table 3). All bacteria exhibited similar level of pathogen growth control at 40 ppm. Pycnidia development was totally prevented at still lower concentrations - at 8 ppm by ARB1 and at 10 ppm by ARB3 and ARB4. With the increase in concentration of extract, growth of *P. azadirachtae* as well as pycnidia production decreased proportionately (Table 3). The results indicated substantial antifungal activity of all the three isolated rhizobacteria against the die-back pathogen *P. azadirachtae*.

Table 2: Amount of ethyl acetate fractions from culture filtrates of three rhizobacteria

Bacteria	Ethyl acetate fractions (mg)
ARB1	79.2
ARB3	83.7
ARB4	70.2

Table 3: Effect of Ethyl acetate fraction of rhizobacteria at different concentrations against *Phomopsis azadirachtae*

Concen -trations	ARB1		ARB3		ARB4	
	Diameter of the mycelial mat (mm)	Number of Pycnidia	Diameter of the mycelial mat (mm)	Number of Pycnidia	Diameter of the mycelial mat (mm)	Number of Pycnidia
Control	90.0 ± 0.93	195 ± 1.48	80.33 ± 0.95	187 ± 1.39	84.0 ± 0.68	239 ± 1.57
2 ppm	79.0 ± 0.89	125 ± 1.34	80.0 ± 0.93	120 ± 1.48	81.0 ± 0.82	158 ± 1.88
4 ppm	59.33 ± 0.88	55 ± 1.41	72.0 ± 0.73	56 ± 1.55	78.66 ± 0.99	97 ± 1.06
6 ppm	36.66 ± 0.80	14 ± 0.86	40.66 ± 0.80	25 ± 1.29	45.66 ± 0.71	34 ± 1.34
8 ppm	26.0 ± 0.86	0	35.33 ± 0.56	19 ± 1.0	27.0 ± 0.77	12 ± 0.93
10 ppm	17.33 ± 0.49	0	23.33 ± 0.76	0	21.66 ± 0.76	0
20 ppm	12.66 ± 0.71	0	12.66 ± 0.71	0	11.0 ± 0.63	0
40 ppm	0	0	0	0	0	0
50 ppm	0	0	0	0	0	0
60 ppm	0	0	0	0	0	0
80 ppm	0	0	0	0	0	0
100 ppm	0	0	0	0	0	0

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

Discussion

Globally phytopathogens cause a serious threat to plant health and productivity. Management of phytopathogens is still mainly attained by the application of chemical products. The natural environment is harmed by these pesticides because they increase environmental pollutants and produce residue build up. Biocontrol of phytopathogens using microorganisms has been found to minimize pollution and problems coupled with synthetic chemicals usage.[12] Microbes in the rhizosphere region, through competition and different antagonistic mechanisms, exhibit significant antimicrobial effects in opposition to plant diseases.[13]

In this study, bacteria have been obtained from *A. paniculata* plant's rhizosphere and first tested against *P. azadirachtae* by the dual culture method followed by agar well diffusion method using the ethyl acetate extract of their culture filtrates. Three bacteria were isolated and named as ARB1, ARB3 and ARB4. All three bacteria showed significant inhibition of *P. azadirachtae* both in the dual culture method as well as in the agar well diffusion method. Dual culturing is a regularly employed preliminary method to test the antagonistic potential of microbes. Many researchers have used this procedure to successfully isolate antifungal bacteria.[14,15,16] The poisoned food method is the frequently used approach to study antifungal activity.[17] Isolation and testing of rhizobacteria from other plants to control phytopathogenic fungi have been reported.[15,16] Rhizosphere of medicinal plants is an excellent habitat for vast number of microorganisms and the rhizobacteria present here are known to serve variety of purposes including plant pathogen control.[18]

Microbes that inhibit phytopathogens are reported to produce various secondary metabolites. Examples are antibiotics and toxins, polyketides (PKs), ribosomal peptides (RPs), and volatile organic compounds (VOCs), & non-ribosomal peptides (NRPs),[13] which provide antagonistic potential to microbes to inhibit phytopathogens. The ethyl acetate extracts of rhizosphere bacteria used in this study might be rich in such secondary metabolites leading to antagonistic activity, but precise understanding of this requires further research. To our knowledge, this is the first report of antifungal activity of *A. paniculata* rhizobacteria against fungal phytopathogens, particularly against *P. azadirachtae*.

Conclusion

This die-back disease is causing a serious problem to the growth of neem trees. This demands immediate attention for effective management. In the current investigation, the rhizobacteria of the *A. paniculata* plant have exhibited effective results against *P. azadirachtae* by completely suppressing the pathogen's growth at low concentrations. Thus, this could provide an efficient, environment-friendly method of controlling *P. azadirachtin* if developed as a biopesticide or as an integrated disease control strategy.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Aavailability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Authors contributions

- **Sheethal Prabakar:** Data collection, Analysis, Writing – original draft
- **Girish Krishna:** Conceptualization, Methodology, Writing - Review and Editing

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