IN VITRO EVALUATION OF ANTAGONISTIC MICROORGANISMS FOR THE CONTROL OF DIE-BACK OF NEEM CAUSAL AGENT *PHOMOPSIS AZADIRACHTAE*

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Abstract: The die-back of neem caused by *Phomopsis azadirachtae* is a devastating disease in India reducing the life span and seed production of neem. Six isolates of antagonistic bacteria and fungi, *Bacillus cereus* (MTCC 430), *B. subtilis* (MTCC 619), *Pseudomonas aeruginosa* (MTCC 2581), *P. oleovorans* (MTCC 617), *Trichoderma harzianum* (MTCC 792) and *T. viride* (MTCC 800) were evaluated against *P. azadirachtae* under *in vitro* conditions. Culture filtrates of all these microorganisms were extracted using ethyl acetate, and the obtained fractions were tested for their antifungal activity against *P. azadirachtae* at different concentrations. Ethyl acetate extracts of *B. subtilis* and *P. aeruginosa* were highly effective and completely inhibited the growth of *P. azadirachtae* at 25 ppm concentration. Both these isolates may be considered as factors for the biological control of die-back of neem.

Key words: *Bacillus subtilis,* biological control, die-back of neem, *Phomopsis azadirachtae, Pseudomonas aeruginosa*

INTRODUCTION

Phomopsis azadirachtae Sateesh, Bhat & Devaki is the causal organism of die-back of neem (Sateesh *et al.* 1997). The diseased trees show characteristic twig blight symptoms and also inflorescence blight and fruit rot. This disease results in a drastic reduction of evergreen canopy and in almost 100% loss of fruit production and thus affecting the availability of seeds (Shankara Bhat *et al.* 1998). The die-back of neem is spreading at an alarming rate reducing the life span and seed production, and thus there is an urgent need for the development of effective management strategies.

Die-back of neem incited by *P. azadirachtae* can be controlled by the application of a systemic fungicide Bavistin (Sateesh 1998; Girish *et al.* 2009). Bavistin completely suppressed mycelial growth, sporulation and conidial germination at 0.25 ppm (Girish *et al.* 2009). Die-back affected neem trees treated with 1% Bavistin 50 WP as foliar spray for four months at intervals of 15 days, completely recovered from the disease. Treatment of neem seeds with Bavistin resulted in death of this seed-borne pathogen. Germination of neem seeds was not affected by Bavistin even at higher concentrations (up to 2000 ppm) (Sateesh 1998). However, the used synthetic fungicide, in general, leads to residue problems and accumulation of toxic pollutants in soil or underground water, and is deleterious for associated soil microbiota (Bunker and Mathur 2001). Plant pathogens develop resistance to synthetic fungicides with continuous exposure (Brent 1995). Carcinogenic, teratogenic, oncogenic and genotoxic properties of synthetic fungicides are known (Carter *et al*. 1984; Dalvi and Whittaker 1995). These aspects resulted in the development of some new plant disease management practices (Agrios 2004).

Since the bio-hazardous nature of fungicides is known, the development of alternative strategies for management of this disease is required. Microbial communities in natural ecosystems through competition, predation or antibiosis, control the abundance of other microorganisms (Bolwerk *et al*. 2005). Biological control of plant diseases using microorganisms provides a possible alternative to the input decrease of agrochemicals in agriculture (Lugtenberg and Bloemberg 2004) and thereby prevents many of associated environmental and ecological problems. This could result in sustainable dis-

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ease management strategy. Owing to the above aspects of developing a biocontrol strategy for die-back of neem, in the present study four bacterial and two fungal antagonists were screened for their antifungal activity against *P. azadirachtae* under *in vitro* conditions.

MATERIALS AND METHODS

Antagonistic microorganisms

The bacterial and fungal antagonists selected for *in vitro* screening against *P. azadirachtae* were *Bacillus cereus* (MTCC 430), *B. subtilis* (MTCC 619), *Pseudomonas aeruginosa* (MTCC 2581), *P. oleovorans* (MTCC 617), *Trichoderma harzianum* (MTCC 792) and *T. viride* (MTCC 800). All cultures were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The *P. aeruginosa* strain, employed in this study, is non-pathogenic to humans (Microbial Type Culture Collection 2000).

All bacterial cultures, except *P. aeruginosa* were first streaked on nutrient agar (Himedia, India) in Petri dishes and a single cell colony was isolated from each culture. A single cell colony of each bacterium was grown on nutrient agar slant and was maintained at 4°C. *P. aeruginosa* was isolated and maintained on King's B medium. The fungal isolates were sub-cultured and maintained on malt extract agar (Himedia, India) at 4°C (Dhingra and Sinclair 1995; Sateesh 1998).

Isolation of ethyl acetate fraction from bacterial culture filtrates (BCF)

The extraction of antifungal ethyl acetate fraction from BCF was carried out as described by Lavermicocca *et al.* (2000). 100 ml of nutrient broth (Himedia, India) in 500 ml Erlenmeyer flask was inoculated with a loopful of 24h-old-culture. Totally 1500 ml of medium was inoculated. 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 culture filtrate was made up to 1.5 l with sterile distilled water, filter sterilized using 0.45 μm membrane filter (Sartorius, Goettingen, Germany) and stored at 4°C.

For extraction, the culture filtrates were concentrated to 10% of their original volume by using a flash evaporator at 50°C (Zhang and Watson 2000) and pH of the BCF (150 ml) was adjusted to 3.6 using 1.0 N HCl. Then the BCF was 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 room temperature to obtain brownish, semi-solid crude extract.

Isolation of ethyl acetate fraction from fungal culture filtrates (FCF)

This was carried out according to Singh *et al.* (2005). 100 ml of potato dextrose broth (PDB, Himedia, India) in 500 ml conical flasks were inoculated with mycelial agar discs taken from the margin of seven-day-old culture of both fungal antagonists separately. Totally 1500 ml of medium was inoculated and all the flasks were incubated at 26±2°C for 25 days. Then the mycelial mats were filtered through Whatman No.1 filter paper, culture filtrates of each fungus were collected separately and concentrated to 10% of their original volume by using a flash evaporator at 50°C. For extraction the volume was made up to 300 ml using sterile distilled water and the culture filtrates were fractionated three times with equal volume of ethyl acetate. The ethyl acetate extracts were combined and evaporated at room temperature to obtain a dark brown, semi-solid crude material.

Bioassay of antifungal activity

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 (Singh *et al.* 2005).

Effect of ethyl acetate fractions of different antagonistic microbial culture filtrates on mycelial growth of *P. azadirachtae*

Ethyl acetate fractions were tested against the pathogen using poison-food technique (Dhingra and Sinclair 1995). Stock solutions of all ethyl acetate fractions were added separately to sterile potato dextrose agar (PDA, Himedia, India) to obtain different concentrations of 25, 50, 100, 250 and 500 ppm. PDA amended with the control solution (500 ppm) 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 *P. azadirachtae.* The inoculated Petri dishes were incubated at 26±2°C with 12 h photoperiod for 10 days. All treatments had four replications and the experiment was repeated three times. Concentration of ethyl acetate fractions required for complete inhibition of mycelial growth was recorded. Mean colony diameter was determined. The colony diameter was compared with the control to measure fungitoxicity. Per cent inhibition (PI) with respect to the control was computed using formula

$$PI = \frac{C - T}{C} \times 100$$

where C is the colony diameter of the control and T is that of the treated combinations.

The comparative studies of ethyl acetate fractions of culture filtrate of *B. subtilis* and *P. aeruginosa*

The ethyl acetate fractions of culture filtrates of *B. subtilis* and *P. aeruginosa* were screened at much lower concentrations *viz.*, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 ppm to test the effect on vegetative growth (mycelial radial growth and dry weight) and the number of pycnidia. For the study of the effect on germ tube, growth at 2.5, 5.0, 10.0, 15.0, 20.0, 25.0 ppm concentrations were tested. All the treatments consisted of four replications and the experiment was repeated three times.

Effect on mycelial growth of *P. azadirachtae*

The experiment was carried out employing the same methodology as described earlier.

Effect on mycelial dry weight of *P. azadirachtae*

Each of 50 ml of PDB amended with ethyl acetate fractions at 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 ppm concentrations were transferred to separate Erlenmeyer flasks. Flasks containing medium with control solution (25 ppm) served as control and all the flasks were inoculated with the five mm mycelial-agar disc taken from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae.* The inoculated flasks were incubated at 26°C and 25 rpm for 20 days. Then the mycelial dry weight was determined using dried mycelial mats with constant weight collected on a preweighed Whatman No.1 filter paper and dried at 70°C in a hot air oven until a constant weight was obtained. All the treatments had four replications and the experiment was repeated three times.

Effect on the number of *P. azadirachtae* pycnidia

PDA amended with different concentrations of the ethyl acetate extracts (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 ppm) were poured to separate Petri dishes (20ml Petri dish^{-1}). The Petri dishes containing media amended with control solution (25 ppm) served as control. All Petri dishes were inoculated with the five mm mycelial-agar disc taken from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae* and were incubated at 26±2°C with 12 h photoperiod for 15 days. All treatments had four replications and the experiment was repeated three times. The number of pycnidia was counted after 15 days of incubation. The bottom area of Petri dishes was divided into six equal parts by diagonally marking the lid with a marking pen. Pycnidia present in each part were counted and mean value was taken as a total count (Sateesh 1998).

Effect on conidial germ tube growth of *P. azadirachtae*

Conidial suspension containing 10^3 conidia per ml of sterile distilled water was prepared and 1.0 ml of this suspension was inoculated to 10 ml of malt extract broth (Himedia, India) containing various concentrations of ethyl acetate extracts (2.5, 5.0, 10.0, 15.0, 20.0, 25.0 ppm) in 100 ml Erlenmeyer flasks. Flasks containing medium with control solution (25 ppm) were inoculated and maintained as control. The flasks were incubated aerobically at 26°C and 25 rpm for 24 h. Then the germ tube growth in each flask was stopped by adding 2.0 ml of 1% lactophenol solution. The germ tube length was measured in microscopic field using a micrometer. Only when the germ tube length was double the conidial length, the conidia were considered as germinated.

Statistical analysis

The following statistical techniques were employed: 't' test, one way/two way ANOVA followed by Tukey's test HSD (Honestly Significant Differences) a = 0.05 using SPSS for windows (version 14.0) evaluation version.

RESULTS

Isolation of ethyl acetate fraction from microbial culture filtrates (BCF and FCF)

The amount of ethyl acetate fractions obtained from the culture filtrates of different antagonistic microorganisms are presented in table 1.

Table 1. Amount of ethyl acetate fractions obtained from culture filtrates of different antagonistic microorganisms

Microorganisms	Ethyl acetate fraction [mg]
Bacillus cereus	501
Bacillus subtilis	477
Pseudomonas aeruginosa	445
Pseudomonas oleovorans	396
Trichoderma harzianum	522
Trichoderma viride	558

Effect of ethyl acetate fractions of different antagonistic microbial culture filtrates on mycelial growth of *P. azadirachtae*

Of the six microbes tested, only two bacterial species *viz., B. subtilis* and *P. aeruginosa* exhibited a complete suppression of mycelial growth of the pathogen at 25 ppm concentration of their ethyl acetate fraction. All other microorganisms (*B. cereus, P. oleovorans, T. harzianum* and *T. viride*) were unable to completely inhibit the mycelial growth of *P. azadirachtae* at 25 ppm of ethyl acetate fraction (Table 2). The ethyl acetate fractions of *B. subtilis* and *P. aeruginosa* showed fungistatic effect at 22.5 ppm and fungicidal effect at 25 ppm (Table 3). A statistically significant difference was observed between the treatments on the growth of pathogen ($p \leq 0.000$).

Table 2. Effect of ethyl acetate extracts of different microbial culture filtrates on the mycelial growth of *P. azadirachtae* at 25 ppm concentration

Microorganisms	Mycelial growth [cm]	Growth inhibition [%]
Control	8.5±0.018 f	0.00±0.00 a
Trichoderma harzianum	5.58±0.040 e	35.63±0.47 b
Trichoderma viride	6.02±0.058 d	29.64±1.48 c
Pseudomonas oleovorans	5.07±0.038 c	41.68±0.44 d
Bacillus cereus	4.43±0.042 b	49.12±0.49 e
Bacillus subtilis	0.00±0.00 a	100.00±0.00 f
Pseudomonas aeruginosa	0.00±0.00 a	100.00±0.00 f

Values are means of three experiments each with four replications±S.E. Figures followed by different superscript letters differ significantly ($p \leq 0.000$) when subjected to Tukey's test HSD (Honestly Significant Differences) ($\alpha = 0.05$)

Table 3. Effect of ethyl acetate extracts of *B. subtilis* and *P. aeruginosa* culture filtrates on the mycelial growth of *P. azadirachtae*

Concentrations of fungicides in ppm	Ethyl acetate extracts of culture filtrates			
	Bacillus subtilis		*Pseudomonas aeruginosa*	
	mycelial growth [cm]	growth inhibition [%]	mycelial growth [cm]	growth inhibition [%]
0.00	8.9±0.037 i	0.00±0.00 a	8.9±0.037 j	0.00±0.00 a
2.5	8.64±0.035 h	2.97±0.40 b	8.33±0.032 i	6.40±0.35 b
5.0	7.92±0.039 g	11.08±0.47 c	7.50±0.032 h	15.73±0.36 c
7.5	6.86±0.047 f	22.95±0.53 d	6.73±0.026 g	24.35±0.29 d
10.0	6.17±0.034 e	30.69±0.38 e	5.85±0.023 f	34.27±0.26 e
12.5	5.49±0.032 d	38.33±0.36 f	5.20±0.019 e	41.55±0.22 f
15.0	4.50±0.029 c	49.47±0.32 g	4.26±0.027 d	52.13±0.30 g
17.5	2.66±0.031 b	69.99±0.43 h	2.30±0.031 c	74.19±0.34 h
20.0	1.72±0.025 a	80.62±0.29 i	1.29±0.018 b	85.55±0.20 i
22.5	0.00±0.00 **a**	100.00±0.00 **j**	0.00±0.00 **a**	100.00±0.00 **j**
25.0	0.00±0.00 **a**	100.00±0.00 **j**	0.00±0.00 **a**	100.00±0.00 **j**

Values are means of three experiments each with four replications±S.E. Figures followed by different superscript letters differ significantly ($p \leq 0.000$) when subjected to Tukey's test HSD (Honestly Significant Differences) ($\alpha = 0.05$)

Effect on mycelial dry weight of *P. azadirachtae*

The ethyl acetate extract of both *B. subtilis* and *P. aeruginosa* totally checked the mycelial growth of *P. azadirachtae* in liquid media at 25 ppm. Inhibition of mycelial growth of the pathogen at different concentrations of the two ethyl acetate extracts is shown in the table 4. A statistically significant difference was observed among the concentrations on the growth of pathogen ($p \leq 0.000$).

Table 4. Effect of ethyl acetate extracts of *B. subtilis* and *P. aeruginosa* culture filtrates on the mycelial dry weight of *P. azadirachtae*

Concentrations of fungicides in ppm	Dry weight of *Phomopsis azadirachtae* [mg±S.E.]	
	Bacillus subtilis	*Pseudomonas aeruginosa*
0.00	315.1±1.41 j	315.1±1.41 j
2.5	279.5±1.78 i	285.6±1.07 i
5.0	218.0±1.41 h	224.5±1.42 h
7.5	163.2±1.12 g	178.2±0.68 g
10.0	133.2±0.90 f	123.1±0.75 f
12.5	90.4±0.60 e	78.8±0.58 e
15.0	53.7±0.51 d	44.7±0.62 d
17.5	28.9±0.41 c	24.4±0.60 c
20.0	4.5±0.15 b	4.1±0.11 b
22.5	0.00±0.00 a	0.00±0.00 a
25.0	0.00±0.00	0.00±0.00 a

Values are means of three experiments each with four replications±S.E. Figures followed by different superscript letters differ significantly ($p \leq 0.000$) when subjected to Tukey's test HSD (Honestly Significant Differences) ($\alpha = 0.05$)

Effect on the number of *P. azadirachtae* pycnidia

The formation of *P. azadirachtae* pycnidia was completely suppressed at 25 ppm of the ethyl acetate fractions of both *B. subtilis* and *P. aeruginosa.* At 20 ppm concentration a few pycnidia, devoided of conidial cirrhi, were produced in both the cases. The effect of different concentrations of these two ethyl acetate extracts on production of the pathogen pycnidia is presented in table 5. A statistically significant difference was observed among the concentrations on pycnidia formation of the pathogen ($p \leq 0.000$).

Table 5. Effect of ethyl acetate extracts of *B. subtilis* and *P. aeruginosa* culture filtrates on pycnidia of *P. azadirachtae*

Concentrations of fungicides in ppm	Number of pycnidia of *Phomopsis azadirachtae* [± S.E.]	
	Bacillus subtilis	*Pseudomonas aeruginosa*
0.00	220.33±1.20 j	220.33±1.20 j
2.5	196.67±1.50 i	203.17±1.66 i
5.0	162.33±1.17 h	182.50±1.34 h
7.5	141.00±1.18 g	169.83±1.35 g
10.0	112.67±1.41 f	142.17±1.22 f
12.5	97.33±0.99 e	110.33±0.76 e
15.0	65.50±1.48 d	85.50±0.99 d
17.5	33.17±1.30 c	51.00±1.41 c
20.0	8.83±0.40 b	17.83±1.05 b
22.5	0.00±0.00 a	0.00±0.00 a
25.0	0.00±0.00 a	0.00±0.00 a

Values are means of three experiments each with four replications±S.E. Figures followed by different superscript letters differ significantly ($p \leq 0.000$) when subjected to Tukey's test HSD (Honestly Significant Differences) ($\alpha = 0.05$)

Effect on conidial germ tube growth of *P. azadirachtae*

Ethyl acetate extracts of both *B. subtilis* and *P. aeruginosa* totally inhibited the germ tube growth at 25 ppm. The suppression of germ tube growth of the conidia at different concentration is presented in the table 6. Conidia lost their fusiform shape and turned into non-germinable oval-shaped structures on exposure to 25 ppm of ethyl acetate fractions of both the bacteria. A statistically significant difference was observed among the concentrations on the conidial germ tube growth of pathogen ($p \leq 0.000$).

Table 6. Effect of ethyl acetate extracts of *B. subtilis* and *P. aeruginosa* culture filtrates on the germ tube growth of *P. azadirachtae*

Concentrations of fungicides in ppm	Germ tube length of *Phomopsis azadirachtae* [μm±S.E.]	
	Bacillus subtilis	*Pseudomonas aeruginosa*
0.00	106.1±0.25 g	106.1±0.25 g
2.5	91.9±0.51 f	96.3±0.52 f
5.0	82.3±0.57 e	85.9±0.53 e
10.0	61.8±0.41 d	56.2±0.56 d
15.0	38.1±0.38 c	32.0±0.37 c
20.0	18.4±0.41 b	12.4±0.48 b
25.0	0.00±0.00 a	0.00±0.00 a

Values are means of three experiments each with four replications±S.E. Figures followed by different superscript letters differ significantly ($p \leq 0.000$) when subjected to Tukey's test HSD (Honestly Significant Differences) ($\alpha = 0.05$)

DISCUSSION

As biological control methods are compatible with sustainable agriculture they are becoming popular (Singh *et al.* 2005). Both bacteria and fungi have proved to be potential antagonists of phytopathogenic fungi (Hajra *et al.* 1992; Lange *et al.* 1993). Several microorganisms secrete distinct secondary metabolites with significant antifungal toxicity (Lange *et al.* 1993).

Among the six antagonistic microorganisms screened, secondary metabolites of *B. subtilis* and *P. aeruginosa* were highly effective against *P. azadirachtae* at comparatively low concentrations. Studies on the effect of ethyl acetate extract on mycelial weight, pycnidial number and germ tube length of the pathogen revealed that both bacterial extracts were highly effective in suppressing growth of the pathogen. Biological control activity of *B. subtilis* and *Pseudomonas* spp., against several *Phomopsis* species was reported (Cubeta *et al.* 1985; Fuchs and Defago 1991; Kita *et al.* 2005; Maurhofer *et al.* 1992). *P. aeruginosa* was employed as active biocontrol agent against many plant pathogens (Anjaiah *et al.* 2003; Krishna Kishore *et al.* 2005; Siddique and Ehteshamul-Haque 2001).

With the increase in the concentration of solvent extract of culture filtrate a significant decrease ($p \leq 0.000$) in the colony diameter, dry weight, pycnidial number and germ tube length were observed. This may be attributed to the exposure of the pathogen to increasing concentrations of antibiotics or other antimycotic secondary metabolites produced by the antagonistic bacteria. *B. subtilis* secretes antifungal antibiotics having broad spectrum activity (Cavaglieri *et al.* 2005; Leclere *et al.* 2005). Production of antibiotics is recognized as a major factor in suppression of plant pathogens by *Pseudomonas* spp. (Dowling and O'Gara 1994; Maurhofer *et al.* 1992). Microorganisms that produce antibiotics are very important in the plant disease management because these antibiotics play a major role in a significant reduction of diseases in the field (Fravel 1988). *Bacillus* spp., produce many other volatile and non-volatile antifungal metabolites (Sharifi-Tehrani *et al.* 2005). *P. aeruginosa* is also known to produce several other antifungal compounds (Audenaert *et al.* 2001; Sunish Kumar *et al.* 2005). With all these characteristics both *B. subtilis* and *P. aeruginosa* serve as promising biocontrol agents.

Singh *et al.* (2005) reported a significant effect of ethyl acetate fraction of *Leptoxyphium axillatum* on per cent spore germination inhibition of plant pathogenic and saprophytic fungi. *B. subtilis* induces morphological abnormalities in the phytopathogenic fungi such as mycelial and conidial deviations (Chaurasia *et al.* 2005). Similar effects were observed in the present study on the germination and morphology of *P. azadirachtae* conidia by ethyl acetate extracts of both bacterial culture filtrates. Induction of morphological abnormalities and inhibition of spore germination are significant effects as the spores are major infective propagules of phytopathogenic fungi (Agrios 2004).

The other organisms *viz., B. cereus, Trichoderma harzianum* and *T. viride* have antagonistic activity against many plant pathogens (Huang *et al.* 2005; Wani 2005), including *Phomopsis* spp. (Thinggaard 1988). But these microorganisms failed to exhibit considerable inhibitory effects against *P. azadirachtae.* This may be due to the inability of these microorganisms to produce potent toxic secondary metabolites that are lethal to *P. azadirachtae.*

Several commercial products based on microorganisms have been developed. However, variability and inconsistency of biocontrol activity are limitations to the large-scale use. In some cases this may result because of sensitivity of the biocontrol agents to environmental factors. These limitations of biocontrol could be overcome by increasing their efficacy by integration of biocontrol with chemical fungicides (Elad 2003).

The tested isolates of *B. subtilis* and *P. aeruginosa* produced agar diffusible, solvent soluble, antimycotic substance against *P. azadirachtae.* The efficacy of the fractions from both *B. subtilis* and *P. aeruginosa* at low concentrations indicates a possibility of their use as safe alternative to chemical fungicides for the effective management of die-back of neem under field conditions and for seed treatment. However, a large scale application needs methodology for large scale production of the extracts and their proper formulation, and also has to be tested in field trials. To overcome the limitations, as mentioned previously, the integration of these natural extracts with other management strategies such as chemical fungicides should be tested. For the registration of biopreparations, before their introduction to the market, a variety of data

about the composition, mammalian toxicity, gene flow assessment, non-target organism hazard assessment including soil organisms, degradation, and other characteristics of the pesticide have to be submitted to the respective agencies. Further research in these areas has the potential to extend the usefulness of the above biopesticides for the effective, eco-friendly management of die-back of neem.

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POLISH SUMMARY

OCENA *IN VITRO* MIKROORGANIZMÓW ANTAGONISTYCZNYCH DO ZWALCZANIA ZGORZELI *AZADIRACHTA INDICA* WYWOŁANEJ PRZEZ *PHOMOPSIS AZADIRACHTAE*

Zgorzel *Azadirachta indicato* wywołana przez *Phomopsis azadirachtae* jest ważną chorobą w Indiach, ograniczającą okres żywotności i produkcję nasion *A. indica*. Sześć izolatów antagonistycznych mikroorganizmów takich jak: *Bacillus cereus* (MTCC 430), *B. subtilis* (MTCC 619), *Pseudomonas aeruginosa* (MTCC 2581), *P. oleovorans* (MTCC 617), *Trichoderma harzianum* (MTCC 792) i *T. viride* (MTCC 800), przetestowano *in vitro* przeciwko *P. azadirachtae.* Filtraty kultur tych sześciu mikroorganizmów ekstrahowano octanem etylu. Otrzymane frakcje octanu etylu testowano pod względem aktywności przeciwgrzybowej. Były one wysoce efektywne w stężeniu 25 ppm i całkowicie inhibitowały wzrost *P. azadirachtae*, gdy były użyte w tym stężeniu. Zarówno *B. subtilus* jak i *P. aeruginosa* mogą być rozważone jako czynniki biologicznego zwalczania zgorzeli *A. indcia.*