



Integrated management of *Phomopsis azadirachtae*, the causal organism of die-back of neem

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ABSTRACT

Phomopsis azadirachtae is the causal organism of die-back of neem. Combinations of two systemic fungicides such as carbendazim and thiophanate-methyl with ethyl acetate extract of culture filtrate of antagonistic bacterium *Bacillus subtilis* were evaluated against *P. azadirachtae* under *in vitro* conditions. The parameters studied were mycelial dry weight, colony diameter, pycnidial formation and the conidial germ tube growth of the pathogen. The effect of these combinations on neem seed germination and seed-borne pathogen was also tested. The results indicated that the combinations tested were effective in inhibiting the growth of the pathogen *in vitro*. The combinations also inhibited the growth of *P. azadirachtae* from die-back infected neem seeds and had no significant negative effect on neem seed germination. These combinations could be utilized for the integrated control of die-back of neem.

Key words: *Phomopsis azadirachtae*, integrated management, *Bacillus subtilis*, carbendazim, thiophanate-methyl

INTRODUCTION

Die-back of neem (*Azadirachta indica* A. Juss.) is caused by *Phomopsis azadirachtae* Sateesh, Bhat & Devaki (Sateesh *et al.*, 1997). This disease is spreading at an alarming rate resulting in the reduction of life expectancy and flower production. The characteristic symptoms of this disease are twig blight, inflorescence blight and fruit rot. This disease results in almost 100% loss of fruit production and drastic reduction in evergreen canopy (Girish and Shankara Bhat, 2008). Neem seeds have many medicinal and biopesticidal ingredients and are the major commercial product of neem. Neem based bio-pesticides are identified has better alternatives to toxic pesticides (Sateesh, 1998). Neem has an important role in economics of India. Establishment of neem plantations is a major aim of neem foundation (Anonemous., 2009). The die-back disease is great obstacle to attain this goal and presently management of this devastating disease is a major task.

Phomopsis. azadirachtae can be controlled by the application of systemic fungicides, carbendazim and thiophanate-methyl (Girish *et al.*, 2009a). But extensive utilization of fungicides can lead to many problems such as adverse effects on beneficial soil microorganisms (Al-Jedabi, 2009) and the risk of development of fungicide resistance by the pathogen (Possiede *et al.*, 2009). This has led to the development of alternative disease control methods, biological control being the most preferred control measure (Sahayaraj and Sathyamoorthy, 2002; Haggag and Mohamed, 2007; Devaki Rani *et al.*, 2009). *Bacillus subtilis* is effective

against *P. azadirachtae* (Girish *et al.*, 2009b). However, biocontrol agents may not function well under certain conditions such as low temperatures and others (Omar *et al.*, 2006). Chalutz and Droby (1997) reported that the lack of consistency is a major drawback of the biocontrol.

These problems with extensive fungicide application and inefficiency of biocontrol agent can be overcome by 'Integrated Disease Management (IDM)' strategy that provides more stable disease control. Integrated Disease Management strategies are at the forefront of ecologically based or bio-intensive pest management (Jacobsen, 1997). Integrated Disease Management provides many procedures that help us to reduce the usage of chemical pesticides (Paroda, 2000; Vasantharaj David, 2008). Jacobsen *et al.* (2004) stated that, "Integrated Disease Management is a sustainable approach to managing pests by combining biological, cultural, physical and chemical in a way that minimizes economic, health and environmental risks". Among these, combination of chemicals and antagonistic microorganisms has received major preference (Budge and Whipps, 2001; Omar *et al.*, 2006; Yildiz *et al.*, 2007; Anand *et al.*, 2009). Fungicides can be applied simultaneously with a biocontrol agent or alternative applications of chemicals and biocontrol agents can be done (Budge and Whipps, 2001). In the present investigations the ethyl acetate extract of culture filtrate of *B. subtilis* was combined with carbendazim and thiophanate-methyl and the effect of these combinations on the growth of *P. azadirachtae* was studied. The effect of these combinations on neem

seed germination and seed-borne pathogen was also studied.

MATERIALS AND METHODS

The bacterial antagonist and fungicides

The bacterial isolate, *Bacillus subtilis* (MTCC 619), was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *B. subtilis* was maintained on nutrient agar medium (Himedia, Mumbai, India) as single cell cultures, at 4°C. The two systemic fungicides tested were carbendazim (50% W.P.) and thiophanate-methyl (70% W.P.). These fungicides and biocontrol agent were selected based on the results obtained from the studies on their effect on growth of *P. azadirachtae*. Carbendazim at 0.25 ppm and thiophanate-methyl at 0.75 ppm completely suppressed the growth of the pathogen (Girish *et al.*, 2009a). The ethyl acetate extract from culture filtrate of *B. subtilis* inhibited the growth of *P. azadirachtae* at 25 ppm (Girish *et al.*, 2009b).

Isolation of ethyl acetate fraction

The extraction of antifungal ethyl acetate fraction from BCF was carried out as per Lavermicocca *et al.* (2000). A loopful of 24h-old-culture of bacteria was inoculated separately to 100 ml of nutrient broth (Himedia, Mumbai, India) taken in 500 ml of Erlenmeyer flask. Totally 10 l of medium was inoculated. All the inoculated 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) and the supernatant was collected. The supernatant was concentrated to 10% of the original volume by using flash evaporator at 50°C (Zhang and Watson, 2000) and filter-sterilized using 0.45µm membrane filter (Sartorius, Goettingen, Germany). The pH of the BCF (1000 ml) was adjusted to 3.6 using 1.0 N HCl and was extracted with equal volume of ethyl acetate for three times. The organic extracts were pooled and evaporated at RT to obtain 2.718 g of brownish, semi-solid crude extract.

Fungicides plus ethyl acetate fraction

The ethyl acetate fraction obtained was dissolved in sterile distilled water containing 0.1% Tween-20 to obtain stock solution (10000 ppm). Sterilized distilled water containing 0.1% Tween-20 was used as control solution (Singh *et al.*, 2005). The stock solutions of each fungicide were prepared using sterile distilled water. All the concentrations of the fungicides are expressed in terms of active ingredient (a.i.). Each fungicide was combined with ethyl acetate extract of bacteria separately as mentioned in table 1 to obtain different concentrations viz. 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E. Based on the results of the

previous work (Girish *et al.*, 2009a) the 0.25 ppm and 0.75 ppm concentrations of carbendazim and thiophanate-methyl respectively were taken as 100% concentration. Similarly 25 ppm concentration was considered as 100% concentration for ethyl acetate extract of *B. subtilis* (Girish *et al.*, 2009b). The tests were carried out using poison-food technique (Dhingra and Sinclair, 1995). All the treatments had four replications and the experiment was repeated thrice.

Effect on mycelial dry weight of *P. azadirachtae*

Potato dextrose broth (50 ml) (Himedia, Mumbai, India) amended with various combinations of fungicides and ethyl acetate fraction at 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E concentrations were transferred to separate 250 ml Erlenmeyer flasks. Flasks containing medium with control solution and without fungicides were maintained as control and all the flasks were inoculated with the five mm mycelial-agar disc drawn from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae*. The inoculated flasks were incubated aerobically at 26°C and 25 rpm for 20 days. Then the mycelial dry weight was determined using dried mycelial mats with constant weight, which were collected on to a preweighed Whatman No.1 filter paper and dried at 70°C in a hot air oven until a constant weight was obtained.

Phomopsis azadirachtae growth parameters

The solutions of fungicides and ethyl acetate extract were added in combinations to potato dextrose agar (PDA, Himedia, Mumbai, India) to obtain final concentrations viz. 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E. PDA amended with the control solution but no fungicides served as control. About 20 ml of all, the treated and untreated PDA were poured into separate Petridishes (9.0 mm diam.). All the Petridishes were inoculated with the five mm mycelial-agar disc drawn 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 ten days. Concentration of combinations of fungicides with ethyl acetate fractions of the bacteria required for complete inhibition of the mycelial growth was noted. Mean colony diameter was found out by measuring linear growth in three directions at right angles. The colony diameter was compared with the control to measure fungitoxicity. The per cent mycelial growth inhibition (PI) with respect to the control was computed from the formula: $PI = C-T/C \times 100$. Where C is the colony diameter of the control and T is that of the treated combinations.

The pycnidial number was counted after 15 days of incubation. The base 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 total count (Sateesh, 1998).

***Phomopsis azadirachtae* conidial germ tube growth**

Conidial suspension having 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, Mumbai, India) containing various combinations of fungicides and ethyl acetate extract (100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 10F: 100E) taken in different 100 ml Erlenmeyer flasks. Flasks containing medium with control solution and without fungicides 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 ceased by adding 2.0 ml of 1% lactophenol solution. The germ tube length was measured under microscopic field using micrometer. Only when the germ tube length was double the conidial length, the conidia were considered as germinated.

Neem seed germination and seed-borne *P. azadirachtae*

The 50F: 50E concentration of each combination of fungicides and ethyl acetate extracts and its multiple concentrations viz., 50F: 50E X 10, 50F: 50E X 50, 50F: 50E X 100, 50F: 50E X 500, were prepared in 100 ml of sterile distilled water. Healthy neem seeds were freshly harvested, hard endocarp was dissected out, thoroughly washed, and surface-sterilized using sodium hypochlorite solution (with 5% available chlorine) for 15 min. Then the seeds were rinsed well in sterile distilled water for five times. Die-back affected neem seeds were collected, thoroughly washed and surface-sterilized as above. 100 seeds were placed in 25 ml of each solution taken in separate 100 ml beakers and were exposed to the solutions for 24 h. Seeds treated with only distilled water served as control. Healthy and diseased neem seeds were treated separately. After treatment 100 seeds (healthy) were germinated by blotter paper and paper towel methods (ISTA, 1999) and incubated for 15 days at RT with natural alternate day and night photoperiod. Each treatment had four replications. Then root length, shoot length and percentage germination were recorded and the vigour index was calculated using the formula given by Abdul-Baki and Anderson (1973). Treated diseased seeds were plated on PDA at the rate of five seeds per plate and incubated for seven days at $26 \pm 2^\circ\text{C}$ with 12 h photoperiod. Each treatment had four replications.

RESULTS

Mycelial growth, pycnidial number and conidial germ tube growth of *P. azadirachtae*

The mycelial growth of *P. azadirachtae* in liquid medium was completely suppressed at all the combinations of fungicides and ethyl acetate extract. The mycelial growth

on solid medium was completely suppressed at all the combinations of fungicides and ethyl acetate extract except 20F: 80E wherein little mycelial radial growth was observed. Mycelial growth on solid media was also observed at 40F: 60E concentrations of combinations of thiophanate-methyl and ethyl acetate extract of *B. subtilis*. The Effect of different concentrations of each combination of fungicides and ethyl acetate extract on mycelial growth of pathogen on solid media is presented in the Table 2. The pycnidial formation and conidial germ tube growth were completely suppressed at all the combinations of fungicides and ethyl acetate extract except 20F: 80E wherein formation of a few pycnidia and little conidial germ tube growth were observed. The number of pycnidia formed at 20F: 80E were 10.33 ± 0.76 (Carbendazim plus ethyl acetate extract) and 18.67 ± 0.61 (Thiophanate-methyl plus ethyl acetate extract) while in control it was 200.17 ± 2.89 (Number \pm S.E.). The pycnidia formed were devoid of conidial cirri. The conidial germ tube length at 20F: 80E was 13.68 ± 0.48 (Carbendazim plus ethyl acetate extract) and 20.22 ± 0.52 (Thiophanate-methyl plus ethyl acetate extract) whereas in control it was 108.03 ± 0.49 ($\mu\text{m} \pm$ S.E.). In all the treatments except 20F: 80E, conidia lost their fusiform shape and turned into non-germinable oval-shaped structures.

Neem seed germination and seed-borne *P. azadirachtae*

Neem seeds treated with the 50F: 50E X 1, 50F: 50E X 10, 50F: 50E X 50, 50F: 50E X 100, 50F: 50E X 500 concentrations of both the combinations for 24 h germinated normally similar to that of control wherein the seeds were only

Table 1. Combinations of fungicides and ethyl acetate extract of *Bacillus subtilis*

Combinations (%)	Carbendazim + <i>B. subtilis</i>	Thiophanate-methyl + <i>B. subtilis</i>
100F: 0E	0.25 ppm: 0	0.75 ppm: 0
80F: 20E	0.20 ppm: 5 ppm	0.60 ppm: 5 ppm
60F: 40E	0.15 ppm: 10 ppm	0.45 ppm: 10 ppm
50F: 50E	0.125 ppm: 12.5 ppm	0.375 ppm: 12.5 ppm
40F: 60E	0.10 ppm: 15 ppm	0.30 ppm: 15 ppm
20F: 80E	0.05 ppm: 20 ppm	0.15 ppm: 20 ppm
0F: 100E	0: 25 ppm	0: 25 ppm

E: Ethyl acetate extract of *B. subtilis* culture filtrate; F: Fungicide

[Based on the results of the previous work (Girish *et al.*, 2009a and b) the 0.25 ppm and 0.75 ppm concentrations of carbendazim and thiophanate-methyl respectively were taken as 100% concentration. Similarly 25 ppm concentration was considered as 100% concentration for ethyl acetate extract of *B. subtilis*].

Table 2. Effect of different combinations of fungicides and ethyl acetate extract of *Bacillus subtilis* on the mycelial growth of *Phomopsis azadirachtae*

Concentrations	Carbendazim + <i>B. subtilis</i>		Thiophanate-methyl + <i>B. subtilis</i>	
	Mycelial Growth (cm ± S.E.)	Growth Inhibition (% ± S.E.)	Mycelial Growth (cm ± S.E.)	Growth Inhibition (% ± S.E.)
Control	8.57 ± 0.040 ^c	0.00 ± 0.00 ^a	8.57 ± 0.040 ^d	0.00 ± 0.00 ^a
100F: 0E	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.00 ± 0.00 ^a	100.00 ± 0.00 ^d
80F: 20E	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.00 ± 0.00 ^a	100.00 ± 0.00 ^d
60F: 40E	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.00 ± 0.00 ^a	100.00 ± 0.00 ^d
50F: 50E	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.00 ± 0.00 ^a	100.00 ± 0.00 ^d
40F: 60E	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.94 ± 0.047 ^b	89.24 ± 0.54 ^c
20F: 80E	1.15 ± 0.021 ^b	86.33 ± 0.30 ^b	2.03 ± 0.086 ^c	76.62 ± 0.99 ^b
0F: 100E	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.00 ± 0.00 ^a	100.00 ± 0.00 ^d

Figures followed by different superscript letters differ significantly by Tukey's HSD (Honestly Significant Differences) [P=0.05]

treated with distilled water. Root length, shoot length, per cent germination and vigour index in different concentrations of each combination of fungicides and ethyl acetate extract are recorded in Table 3. The growth of *P. azadirachtae* was completely inhibited in all the treatments whereas the untreated control seeds showed almost 90% incidence of *P. azadirachtae*. Few treated seeds even showed a little germination.

DISCUSSION

In this study, systemic fungicides carbendazim and thiophanate-methyl were combined with ethyl acetate extract of culture filtrate of *B. subtilis* and tested *in vitro*

against *P. azadirachtae*. *In vitro* agar plate or nutrient broth based experiments are often used as test systems to determine potential tolerance of fungi to pesticides (Fernando and Linderman, 1994). Carbendazim, thiophanate-methyl and *B. subtilis* are effective against *P. azadirachtae* (Girish *et al.*, 2009a and b). Systemic fungicides carbendazim and thiophanate-methyl are known to control many plant diseases (Lukose *et al.*, 2007; Patel, 2009). *Bacillus subtilis* was utilized as biocontrol agent to manage many plant diseases (Abo-Elnaga, 2006; Al-Jedabi, 2009). Integration of biological control agents and chemical fungicides is the widely accepted and practiced IDM strategy (Budge and Whipps, 2001).

Table 3. Effect of 50F: 50E concentration of combinations of fungicides and ethyl acetate extract of *Bacillus subtilis* on germination of neem seeds

Concentrations	Root Length (cm ± S.E.)	Shoot Length (cm ± S.E.)	Percentage Germination ± S.E.	Vigour Index ± S.E.
Control	11.01 ± 0.058	3.81 ± 0.080	89.38 ± 0.46	1330.76 ± 12.96
A X 1	10.84 ± 0.046	3.76 ± 0.038	88.00 ± 0.65	1274.85 ± 11.25
A X 10	10.55 ± 0.042	3.64 ± 0.032	87.38 ± 0.46	1239.71 ± 10.12
A X 50	10.15 ± 0.060	3.49 ± 0.040	86.50 ± 0.33	1179.74 ± 10.50
A X 100	10.00 ± 0.060	3.28 ± 0.037	85.50 ± 0.42	1135.08 ± 10.69
A X 500	9.80 ± 0.057	3.11 ± 0.030	84.75 ± 0.31	1094.43 ± 8.91
B X 1	10.93 ± 0.045	3.83 ± 0.037	88.00 ± 0.68	1299.94 ± 11.25
B X 10	10.69 ± 0.030	3.75 ± 0.042	87.50 ± 0.42	1263.34 ± 8.25
B X 50	10.33 ± 0.037	3.58 ± 0.031	87.00 ± 0.46	1209.30 ± 6.85
B X 100	10.11 ± 0.035	3.46 ± 0.042	85.88 ± 0.35	1165.66 ± 2.74
B X 500	9.79 ± 0.035	3.31 ± 0.030	85.38 ± 0.38	1118.46 ± 7.45

[A - Carbendazim; *Bacillus subtilis* (50F: 50E); B - Thiophanate- methyl; *Bacillus subtilis* (50F: 50E)]. The data was subjected to Tukey's HSD (Honestly Significant Differences) [P = 0.05].

Integration of biological control agent with chemical fungicides reduces the amount of fungicides to be applied minimizing the associated residual problems. This also helps to overcome biocontrol limitations and to improve its efficacy providing a reliable disease control that cannot be provided by the biocontrol agent alone (Elad, 2003; Omar *et al.*, 2006).

The combinations of each chemical with biocontrol extract were significantly effective against the growth of *P. azadirachtae*. These combinations in all the concentrations tested, totally suppressed the sporulation and germination of spores of the pathogen, and except 20F: 80E and 40F: 60E completely inhibited the vegetative growth. The results of present studies are in conformity with the previous findings about integration of *B. subtilis* and chemical fungicides (Korsten *et al.*, 1997; Kondoh *et al.*, 2001). The 20F: 80E concentration of all the combinations and 40F: 60E concentration of combinations of thiophanate-methyl and ethyl acetate extract of *B. subtilis* showed higher toxicity rate against *P. azadirachtae* in broth medium than agar medium. These results were in agreement with that of Ko *et al.* (1976). They reported that fungicides generally were more effective against fungal growth in liquid than in agar medium. At low concentration carbendazim causes abnormalities in germ tube (Wang *et al.*, 1995). Morphological abnormalities such as mycelial and conidial deviations are induced by *B. subtilis* in phytopathogenic fungi (Chaurasia *et al.*, 2005). In the present study, the combinations of fungicides and ethyl acetate extract produced similar effects on the germination and morphology of *P. azadirachtae* conidia revealing the efficient antifungal activity of the combinations evaluated against *P. azadirachtae*.

In the integrated management strategies employing combinations of chemicals and biocontrol agents, the incompatibility between chemical pesticides and microbial antagonists may be a major setback (Omar *et al.*, 2006). For the success of IDM compatibility between these two is highly required which results in the survival and effective activity of a microbe at an environment in the presence of a chemical (Pandey *et al.*, 2006). Isolation of secondary metabolites having antagonistic activity from biocontrol microorganisms and combining them in a known concentration with low concentrations of fungicides would help to overcome this problem. Identification and utilization of microbial products for plant disease control is an integral part of sustainable agriculture (Haggag and Mohamed, 2007; Hem Saxena, 2008). The control effect of such combinations can be attributed to the synergistic effect of the combined treatments. Such synergistic effect was observed between fungicides and ethyl acetate extract of

B. subtilis in this study which resulted in effective control of the pathogen *in vitro*.

Germination of seeds is used as bioassay to demonstrate the toxic effect of fungicides or biocontrol extracts on the host plant (Nithyameenakshi *et al.*, 2006; Devaki Rani *et al.*, 2009). Systemic and non-systemic fungicides influence the germination of seeds (Maude, 1996). Thus knowledge of phytotoxicity of fungicides or any combinations on host plants is necessary before utilized for field application. In the present study, neem seeds treated with the combinations of carbendazim and thiophanate-methyl with ethyl acetate extract of *B. subtilis* showed significant germination in comparison with control. Exposure to higher concentration (50F: 50E X 100 and above) did not inhibit germination but only delayed the initiation of germination wherein germination occurred after five days. This shows that these combinations are non-toxic to the neem tissues at concentrations that may be used in the field. *P. azadirachtae* is seed-borne and the pathogen transmission and disease spread can occur through seeds (Sateesh and Shankara Bhat, 1999; Girish *et al.*, 2009c). Seed treatment is an effective method to suppress the pathogen growth in seeds (Bharath *et al.*, 2006). The combinations of fungicides and biocontrol extract used in the present study completely inhibited the growth of the *P. azadirachtae* in neem seeds and can be used for neem seed treatments. These results are in accordance with Sateesh (1998) wherein the neem seeds were treated with bavistin and evaluated for germination and growth of *P. azadirachtae* from seeds.

Owing to the results of present investigations, the treatments with 50F: 50E concentration of combinations of carbendazim and thiophanate-methyl with culture filtrate extract of *B. subtilis* could be potential integrated control measure against *P. azadirachtae*.

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