African Journal of Microbiology Research Vol. 6(2), pp. 385-392, 16 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1154
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Synergistic effect of combinations of fungicides and bacterial extracts against *Phomopsis azadirachtae* causing die-back of neem

K. Girish[1,2]*, S. Shankara Bhat[1,3] and K. A. Raveesha[1,4]

[1]Department of Studies in Microbiology, Manasagangotri, University of Mysore, Mysore – 570 006, India.
[2]Postgraduate Department of Microbiology, Maharani's Science College for Women, JLB Road, Mysore - 570 005, India.
[3]Labland Biodiesel Private Limited, # 98, 7th main, Jayalakshmipuram, Mysore -570 012, India.
[4]Department of Studies in Botany, Manasagangotri, University of Mysore, Mysore - 570 006, India.

Accepted 5 December, 2011

***Phomopsis azadirachtae* causes die-back of neem and this disease is presently a major devastating disease of neem in India, resulting in the reduction of life expectancy and flower production. Development of effective, eco-friendly management strategies against this disease is most important. Two systemic fungicides carbendazim and thiophanate- methyl were combined with ethyl acetate extract of antagonistic bacteria *Pseudomonas aeruginosa* culture filtrate at different concentrations *viz*., 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F:100E and evaluated against *P. azadirachtae* under *in vitro* conditions. The parameters studied were colony diameter, mycelial dry weight, pycnidial formation and the 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: Die-back of neem, *Phomopsis azadirachtae*, integrated management, *Pseudomonas aeruginosa,* carbendazim, thiophanate-methyl.

INTRODUCTION

Neem (*Azadirachta indica* A. Juss) commonly known as 'Indian lilac' or 'Margosa', is a native tree to India. Neem finds many applications in the fields of pest management, environment protection and medicine. India stands first in neem seed production which is the major commercial product of neem having many medicinal and biopesticidal compounds. The die-back of neem caused by *P. azadirachtae* (Sateesh et al., 1997) is spreading at an alarming rate resulting 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 diseased trees show characteristic twig blight symptoms and also inflorescence blight and fruit rot.

Fungal diseases of plants are primarily controlled by the application of fungicides (Maloy, 1993). Systemic fungicides carbendazim and thiophanate-methyl are effective against *P. azadirachtae* (Girish et al., 2009a). However, extensive utilization of fungicides adversely affects the components of ecosystem (Rathmell, 1984). It leads to accumulation of toxic pollutants in soil or under-ground water harming associated soil microbiota (Bunker and Mathur, 2001) and development of resistance to synthetic fungicides by plant pathogens (Brent, 1995). There is an increasing awareness that pesticides and

*Corresponding author. E-mail: girishk77@yahoo.com. Tel: +91-9743665772.

fertilizers cause damage to the environment and affect human health (Perkins and Patterson, 1997). As a consequence, there is a trend toward finding eco-friendly alternative plant disease management approaches to minimize the use of fungicides (Mass and Galletta, 1997). In recent years, biological control of phytopathogens has received major attention as a promising alternative to chemical control. Control of plant pathogens by biological means is environmentally advantageous in comparison to chemical control methods that have many risks on human health and environment (Nautiyal, 2001). *P. aeruginosa* is effective against *P. azadirachtae* (Girish et al., 2009b). However, the biocontrol agents are frequently found to be ineffective because of microbial competition or adverse environmental conditions (Lazarovits and Nowak, 1997; Omar et al., 2006) and the lack of consistency is a major drawback of the biocontrol (Chalutz and Droby, 1997).

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. Combinations of two or more methods provide more protection than each component alone (Ehteshamul-Haque et al., 1995). Integrated management has potential to increase the durability of resistance through reduction of pathogen population size and imposition of disruptive selection (Mundt et al., 2002). IDM is a flexible, multidimensional approach to disease control utilizing a range of control components such as cultural, biological and chemical strategies needed to hold diseases below damaging economic threshold without damaging the agrosystem (Papavizas and Lewis, 1988). Integration of bioagents and systemic fungicides is one such management strategy that is widely accepted and practiced (Budge and Whipps, 2001). The efficiency of the biocontrol agent improves when combined with a fungicide (Elad, 2003), which results in a reliable disease control that cannot be provided by the biocontrol agent alone (Omar et al., 2006). The amount of fungicides to be applied is reduced minimizing the associated residual problems. There are reports of integration of *P. aeruginosa* with fungicides for the management of plant diseases (Krishna et al., 2005a and b).

In the present investigations the ethyl acetate extract of culture filtrate of *P. aeruginosa* 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 growth of seed-borne pathogen was also studied.

MATERIALS AND METHODS

The bacterial antagonist and fungicides

The antagonistic bacterial isolate, *P. aeruginosa* (MTCC 2581), used in this study was procured from microbial type culture collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacteria was maintained on King's B medium (Himedia, Mumbai, India), as single cell culture, at 4°C. The systemic fungicides tested were two benzimidazole fungicides, carbendazim (50% W.P.) and thiophanate-methyl (75% 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* (Girish et al., 2009 a and b).

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

The extraction of antifungal ethyl acetate fraction from BCF was carried out as per Lavermicocca et al. (2000). 100 ml of King's B broth (Himedia, India) in 500 ml Erlenmeyer flask was inoculated with a loopful of 24 h-old-culture. 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. Volume of culture filtrate was made up to 10 l with sterile distilled water, filter-sterilized using 0.45 µM membrane filter (Sartorius, Goettingen, Germany) and stored at 4°C. For extraction, 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 0.45 µm membrane filter (Sartorius, Goettingen, Germany). The pH of the BCF (1000 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 filtrate were pooled and evaporated at room temperature to obtain 2.579 g of brownish, semi-solid crude extract.

Effect of combinations of fungicides and ethyl acetate fraction of the bacteria on the growth of *P. azadirachtae*

The ethyl acetate fraction obtained was dissolved in sterile distilled water containing 0.1% Tween-20 to obtain stock solution of 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 bacterium separately as mentioned in Table 1 to obtain different concentrations, namely 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F:100E, considering 0.25 and 0.75 ppm concentrations of carbendazim and thiophanate-methyl respectively as 100% and 25 ppm concentration of ethyl acetate extract of *P. aeruginosa* as 100%, based on the results of the previous work (Girish et al., 2009 a and b). The tests were carried out using poison-food technique (Dhingra and Sinclair, 1995). All the treatments had four replications and the experiments were repeated thrice.

Effect on mycelial growth of *P. azadirachtae*

The solutions of fungicides and ethyl acetate extract were added in combinations to potato dextrose agar (PDA, Himedia, Mumbai, India) to obtain final concentrations, namely 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F:100E. PDA amended with control solution but without fungicides served as control. About 20 ml of the treated and untreated PDA was poured into separate Petri-dishes (90 mm diam.). All the petri-dishes were inoculated with the five mm mycelia 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 10 days. Concentration of combinations of fungicides with ethyl acetate fraction of the bacteria

Table 1. Combinations of fungicides (Carbendazim and Thiophanate-methyl) and ethyl acetate extract of *P. aeruginosa.*

Combinations (%)	Concentrations of fungicides and ethyl acetate extract of *P. aeruginosa*	
	Combination of carbendazim with *P. aeruginosa*	Combination of thiophanate-methyl with *P. aeruginosa*
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	0.45ppm: 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 microbial culture filtrate; F: Fungicide (Based on the results of the previous work (Girish et al., 2009 a and b) the 0.25 and 0.75 ppm concentrations of carbendazim and thiophanate-methyl respectively were taken as 100%. Similarly 25 ppm concentration was considered as 100% for ethyl acetate extract of *P. aeruginosa*).

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

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

Where, C is the colony diameter of the control and the T is that of the treated ones.

Effect on mycelial dry weight of *P. azadirachtae*

50 ml of potato dextrose broth (Himedia, Mumbai, India) amended with various combinations of fungicides and ethyl acetate fraction, namely 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E concentrations separately were transferred to separate 250 ml Erlenmeyer flasks. Flasks containing medium amended with control solution but without fungicides were maintained as control. Each flask was inoculated with the five mm mycelia disc drawn from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae* and incubated in a controlled environment incubator shaker at 26°C and 25 rpm for 20 days. After the incubation period mycelial fragments were 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. Then the mycelial dry weight was determined.

Effect on pycnidial number of *P. azadirachtae*

Petri dishes containing 20 ml of PDA amended with different combinations of fungicides and ethyl acetate extract (100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E) were inoculated with the five mm mycelia disc drawn from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae.* Petri dishes with the media amended with control solution, but without fungicides were inoculated and maintained as control. All the Petri dishes were incubated at 26 ± 2°C with 12 h photoperiod for 15 days. After the incubation period total numbers of pycnidia present were counted. 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).

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

10 ml of malt extract broth (Himedia, Mumbai, India) taken in different 100 ml Erlenmeyer flasks were amended with various combinations of fungicides and ethyl acetate extract (100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E). Conidial suspension having 10^3 conidia per ml of sterile distilled water was prepared and 1.0 ml of this suspension was inoculated to each flask. Flasks containing medium with control solution but no fungicides were inoculated and maintained as control. The flasks were incubated in a controlled environment incubator shaker 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 under microscopic field using micrometer. The conidia were considered as germinated only when the germ tube length was double the conidial length.

Effect on germination of neem seeds

The 50F: 50E concentration of each combination of fungicides and ethyl acetate extract 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. 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 only with distilled water served as control. After treatment the 100 seeds were germinated by blotter paper and paper towel methods (ISTA, 1993), incubating 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).

Effect on seed-borne *P. azadirachtae*

The 50F: 50E concentration of each combination of fungicides and ethyl acetate extract 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. Die-back affected neem seeds were thoroughly washed and surface-sterilized as above. 100 seeds were placed in 25 ml of each solution taken in separate

Table 2. Effect of different combinations of fungicides and ethyl acetate extract of *Pseudomonas aeruginosa* on the mycelial growth of *P. azadirachtae.*

Concentrations	Combination of fungicides and ethyl acetate extract of *P. aeruginosa*			
	Carbendazim with *P. aeruginosa*		Thiophanate-methyl with *P. aeruginosa*	
	Mycelial growth (cm^s)	Growth inhibition (%)	Mycelial growth (cm^s)	Growth inhibition (%)
0	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	0.74 ± 0.030^b	91.55 ± 0.34^c
20F: 80E	1.24 ± 0.038^b	85.67 ± 0.43^b	1.88 ± 0.043^c	78.37 ± 0.63^b
0F: 100E	0.00 ± 0.00^a	100.00 ± 0.00^c	0.00 ± 0.00^a	100.00 ± 0.00^d

Values are means of three experiments and each with four replications ± S.E. Figures followed by different superscript letters differ significantly when subjected to Tukey's honestly significant differences (HSD) [α = 0.05].

Table 3. Effect of different combinations of fungicides and ethyl acetate extract of *P. aeruginosa* on the pycnidial number of *P. azadirachtae.*

Concentrations	Number of pycnidia of *P. azadirachtae* (± S.E.)	
	Combination of fungicides and ethyl acetate extract of *P. aeruginosa*	
	Carbendazim with *P. aeruginosa*	Thiophanate-methyl with *P. aeruginosa*
0	200.17 ± 2.89^c	200.17 ± 2.89^c
100F: 0E	0.00 ± 0.00^a	0.00 ± 0.00^a
80F: 20E	0.00 ± 0.00^a	0.00 ± 0.00^a
60F: 40E	0.00 ± 0.00^a	0.00 ± 0.00^a
50F: 50E	0.00 ± 0.00^a	0.00 ± 0.00^a
40F: 60E	0.00 ± 0.00^a	0.00 ± 0.00^a
20F: 80E	12.00 ± 0.58^b	21.50 ± 0.88^b
0F: 100E	0.00 ± 0.00^a	0.00 ± 0.00^a

Values are means of three experiments and each with four replications ± S.E. Figures followed by different superscript letters differ significantly when subjected to Tukey's honestly significant differences (HSD) [α = 0.05].

100 ml beakers and were exposed to the solutions for 24 h. Seeds treated with only distilled water served as control. After treatment they were plated on PDA at the rate of five seeds per plate and incubated for seven days at 26 ± 2°C with 12 h photoperiod. Each treatment had four replications.

Statistical analysis

Statistical techniques employed were –'t' test, one way / two way ANOVA followed by Tukey's HSD (honestly significant differences) [α = 0.05] using SPSS for windows (version 14.0) evaluation version.

RESULTS

Effect on mycelial growth, pycnidial number and conidial germ tube growth of *P. azadirachtae*

The mycelial growth, pycnidial formation of *P. azadirachtae* on solid medium and germ tube growth were completely suppressed at all the combinations of fungicides and ethyl acetate extract except 20F: 80E wherein little mycelial radial growth, formation of a few pycnidia and germ tube growth were observed. Mycelial growth on solid media was also observed at 40F: 60E concentration of combinations of thiophanate-methyl and ethyl acetate extract of *P. aeruginosa*. The mycelial growth of *P. azadirachtae* in liquid medium was completely suppressed at all the combinations of fungicides and ethyl acetate extract. The pycnidia formed were devoid of conidial cirri. In all the treatments except 20F: 80E, conidia lost their fusiform shape and turned into non-germinable oval-shaped structures. The Effect of different concentrations of each combination of fungicides and ethyl acetate extract on the mycelial growth on solid media, pycnidia formation and germ tube growth of the pathogen are mentioned in Tables 2, 3 and 4 respectively.

Table 4. Effect of different combinations of fungicides and ethyl acetate extract of *P. aeruginosa* on the germ tube growth of *P. azadirachtae.*

Concentrations	Germ tube length of *P. azadirachtae* (µm ± S.E.)	
	Combination of fungicides and ethyl acetate extract of *P. aeruginosa*	
	Carbendazim with *P. aeruginosa*	Thiophanate-methyl with *P. aeruginosa*
0	108.03 ± 0.49^{c}	108.03 ± 0.49^{c}
100F: 0^{E}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
80F: 20^{E}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
60F: 40^{E}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
50F: 50^{E}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
40F: 60^{E}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
20F: 80^{E}	16.52 ± 0.82^{b}	21. 57 ± 0.80^{b}
0F: 100^{E}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are means of three experiments and each with four replications ± S.E. Figures followed by different superscript letters differ significantly when subjected to Tukey's honestly significant differences (HSD) [α = 0.05].

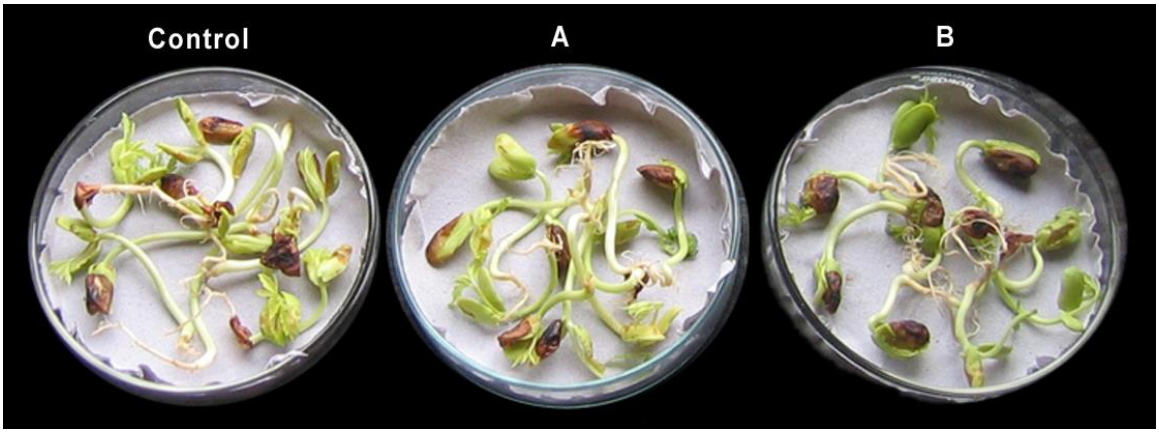

Figure 1. Effect of different combinations of systemic fungicides and microbial ethyl acetate extract on neem seed germination at 50F: 50E X 500 concentration.

Effect on germination of neem seeds

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 all the combinations for 24 h germinated normally similar to that of control seeds that were treated only with distilled water (Figure 1). 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 5.

Effect on seed-borne *P. azadirachtae*

In all the treatments the growth of *P. azadirachtae* was completely inhibited whereas the untreated control seeds showed almost 90% incidence of *P. azadirachtae*. Few treated seeds even showed a little germination (Figure 2).

DISCUSSION

Effective disease control in plants is rarely achieved by using a single control method, and integrated control thus becomes most important (Bruehl, 1989). Jacobsen et al. (2004) stated that, "IDM is a sustainable approach to managing pests by combining biological, cultural, physical and chemical in a way that minimizes economic, health and environmental risks".

In the present study, systemic fungicides carbendazim and thiophanate-methyl were combined with ethyl acetate extract of culture filtrate of *P. aeruginosa* 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). Systemic fungicides carbendazim and thiophanate-methyl are known to control many plant diseases (Meena and Shah, 2005; Ponmurugan et al., 2006). *P. aeruginosa* was

Table 5. Effect of different combinations of fungicides and ethyl acetate extract of *P. aeruginosa* on the germination of neem seeds.

Combinations	Concentrations	Root length (cm)	Shoot length (cm)	Percentage germination	Vigour index
Control	0	11.01 ± 0.058	3.81 ± 0.080	89.38 ± 0.46	1330.76 ± 12.96
Carbendazim: *P. aeruginosa* (50F: 50E) A	A X 1	11.00 ± 0.060	3.80 ± 0.057	88.75 ± 0.59	1313.85 ± 16.54
	A X 10	10.85 ± 0.042	3.75 ± 0.046	87.88 ± 0.40	1281.96 ± 10.66
	A X 50	10.49 ± 0.030	3.65 ± 0.042	87.38 ± 0.38	1235.36 ± 9.78
	A X 100	10.15 ± 0.050	3.49 ± 0.030	86.38 ± 0.42	1177.91 ± 6.54
	A X 500	9.88 ± 0.031	3.24 ± 0.046	85.63 ± 0.26	1122.73 ± 5.09
Thiophanate- methyl: *P. aeruginosa* (50F: 50E) B	B X 1	10.88 ± 0.031	3.78 ± 0.031	87.75 ± 0.31	1285.55 ± 6.92
	B X 10	10.59 ± 0.030	3.65 ± 0.042	87.25 ± 0.45	1242.20 ± 8.11
	B X 50	10.21 ± 0.030	3.53 ± 0.045	85.63 ± 0.38	1176.25 ± 7.10
	B X 100	9.94 ± 0.050	3.45 ± 0.042	84.88 ± 0.30	1136.21 ± 6.40
	B X 500	9.69 ± 0.040	3.19 ± 0.040	84.38 ± 0.46	1086.19 ± 4.61

Values are means of four replications ± S.E. The data was subjected to Tukey's honestly significant differences (HSD) [α = 0.05].

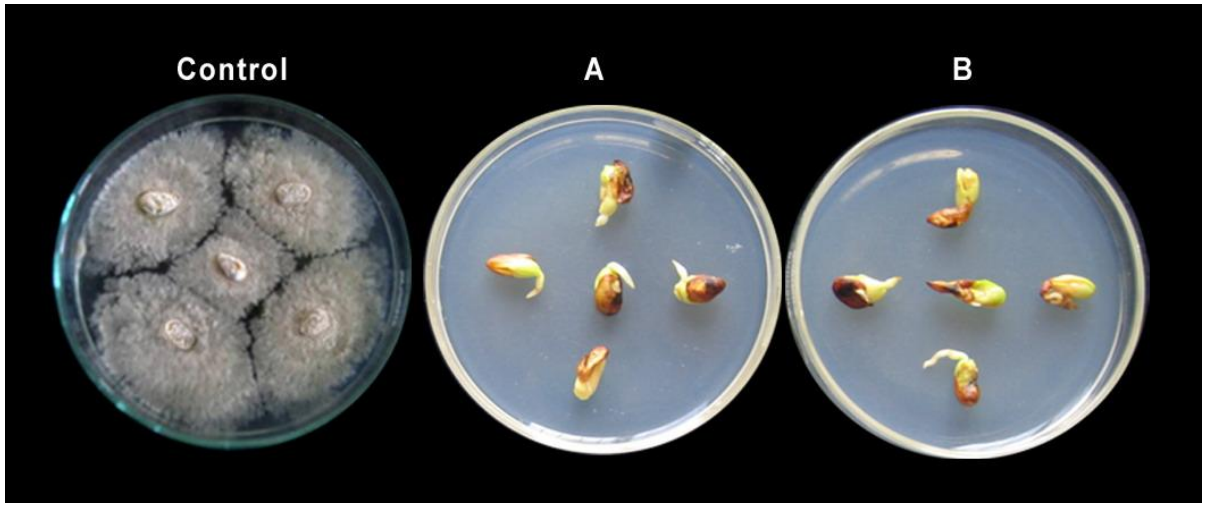

Figure 2. Effect of different combinations of systemic fungicides and microbial ethyl acetate extract on seed-borne *P. azadirachtae* at 50F: 50E X 1 concentration. In both figure 1 and figure 2: A, Seeds treated with combination of carbendazim and ethyl acetate extract of *P. aeruginosa*; B, Seeds treated with combination of thiophanate-methyl and ethyl acetate extract of *P. aeruginosa*; F: Systemic fungicide; E: Ethyl acetate extract of *P. aeruginosa* (Similar results were obtained with all the concentrations tested *viz*., 50F: 50E X 1, 50F: 50E X 10, 50F: 50E X 50, 50F: 50E X 100, 50F: 50E X 500).

employed as active biocontrol agent against many plant pathogens (Siddiqui and Ehteshamul-Haque, 2001; Sunish et al., 2005). Integration of chemicals and antagonistic microorganisms for plant disease management were reported by various workers (Deepak and Dubey., 2001; Kiewnick et al., 2001).

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. Similar results were reported about integration of *P. aeruginosa* and chemical fungicides (Krishna et al., 2005a and b). *P. aeruginosa* GSE18 in association with thiram controlled collar rot disease of groundnut caused by *Aspergillus niger* (Krishna et al., 2005a). Combination of *P. aeruginosa* GSE18 and chlorothalonil resulted in significant reduction of late leaf spot of groundnut disease severity (Krishna et al., 2005b). The 20F: 80E concentration of both the combinations and 40F: 60E concentration of combination of thiophanate-methyl and ethyl acetate extract of *P. aeruginosa* 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), who reported that fungicides generally were more effective against fungal growth in liquid than in agar medium.

Carbendazim causes abnormalities in germ tube at low concentration (Wang et al., 1995). Chaurasia et al. (2005) reported induction of morphological abnormalities in phytopathogenic fungi by *B. subtilis* such as mycelial and conidial deviations. In the present study, the combinations of fungicides and ethyl acetate extract of *P. aeruginosa* produced similar effects on the germination and morphology of *P. azadirachtae* conidia. Such effects that inhibit spore germination are significant as the spores are major infective propagules of phytopathogenic fungi (Agrios, 2004).

In the integrated management strategies wherein combinations of chemicals and biocontrol agents are used, the incompatibility between these two may be a major setback (Omar et al., 2006). For the success of IDM the survival and effective activity of a microbe at an environment in the presence of a chemical is most important. Isolation of antimicrobial secondary metabolites from antagonistic microorganisms and combining them in a known concentration with low concentrations of fungicides would help to overcome this problem. The control effect of such combinations can be attributed to the synergistic effect of the combined treatments. In present investigations, effective control of the pathogen observed *in vitro* may be the result of synergistic effect between fungicides and ethyl acetate extract of *P. aeruginosa*.

Before field application, it is necessary to know the phytotoxicity of fungicides or any combinations on host plants. Germination of seeds is used as bioassay to demonstrate the toxicity of fungicides or biocontrol extracts on the host plant (Dalvi et al., 1972; Chauhan et al., 1997). The germination of seeds is influenced by systemic and non-systemic fungicides (Maude, 1996). Some fungicides adversely affect the seed germination (Devaki, 1991) and reduce the yield. In the present study, neem seeds treated with the combinations of carbendazim and thiophanate-methyl with ethyl acetate extract of *P. aeruginosa* showed significant germination in comparison with control. Exposure to higher concentration (50F: 50E X 100 and above) did not inhibit the 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 to be used in the field.

P. azadirachtae is seed-borne and seed-transmitted (Sateesh and Shankara Bhat, 1999). Seed treatment is the best method to suppress the pathogen in seeds. Punam Singh et al. (1999) reported effective control of seed mycoflora of some forest trees including *Azadirachta indica* (neem) by the seed treatment with carbendazim. Seed treatment with a combination of *Pseudomonas aureofaciens* (Agat-25K) and iprodione (Rovral) increased the disease resistance of sunflower plants against *Phomopsis* sp. (*Diaporthe helianthi*) which in turn improved plant growth and increased yield (Begunov et al., 2000). Similarly, complete inhibition of *P. azadirachtae* growth in neem seeds was observed on treatment with the combinations of fungicides and biocontrol extract used in the present study and thus could 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 concentrations of combinations of carbendazim and thiophanate-methyl with culture filtrate extract of *P. aeruginosa* could be potential integrated control measure against *P. azadirachtae*.

ACKNOWLEDGEMENTS

The authors thank University of Mysore for facilities. K. Girish acknowledges DST, New Delhi for financial support.

REFERENCES

Abdul-Baki AA, Anderson JP (1973). Vigour determination in soybean seed by multiple criteria. Crop Sci., 13: 630-633.

Agrios GN (2004). Plant Pathology; Fourth Edition. Academic Press, New York, USA.

Begunov II, Strelkov VD, Samsonov VG (2000). Agat-25K and *Phomopsis* on sunflower. Zashchita- i- Karantin- Rastenii, 5: 25.

Brent KJ (1995). Fungicide resistance in crop pathogens: How can it be managed? In: GIFAP, FRAC Monograph No.1. Brussels, Belgium: Global Crop Protection Federation.

Bruehl GW (1989). Integrated control of soil-borne plant pathogens: An overview. Can. J. Pl. Pathol., 11: 153-157.

Budge SP, Whipps JM (2001). The potential for integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using *Coniothyrium minitans* and reduced fungicide application. Phytopathology, 91: 221–227.

Bunker RN, Mathur K (2001). Integration of biocontrol agents and fungicide for suppression of dry root rot of *Capsicum frutescens*. J. Mycol. Pl. Pathol., 31: 330-334.

Chalutz E, Droby S (1997). Biocontrol of postharvest diseases. In: Boland GJ, Kuykendall LD (eds) Plant- Microbe Interactions and Biological control. New York: Marcel Dekker, pp. 157-170.

Chauhan RS, Singh BM, Develash RK (1997). Effect of toxic compounds of *Exserohilum turcicum* on chlorophyll content, callus growth and cell viability of susceptible and resistant inbred lines of maize. J. Phytopathol., 145: 435-440.

Chaurasia B, Pandey A, Palni LM, Trivedi P, Kumar B, Colvin N (2005). Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi *in vitro*. Microbiol. Res., 160: 75-81.

Dalvi RR, Singh B, Salunkhe DK (1972). Influence of selected pesticides on germination and associated metabolic changes in wheat and mung bean seeds. J. Agric. Food Chem., 20: 1000-1003.

Deepak K, Dubey SC (2001). Management of color rot of pea by the integration of biological and chemical methods. Indian Phytopath., 54: 62-66.

Devaki NS (1991). Studies on *Pythium* and *Phytophthora* on tobacco in Karnataka. PhD dissertation, University of Mysore, India.

Dhingra OD, Sinclair JB (1995). Basic plant pathology methods; Second Edition. CRC Press, Boca Raton, Florida, USA.

Ehteshamul-Haque S, Abid M, Ghaffar A (1995). Efficacy of *Bradyrhizobium* sp., and *Paecilomyces lilacinus* with oil cakes in the control of root rot of mungbean. Trop. Sci., 35: 294-299.

Elad Y (2003). Biocontrol of foliar pathogens: mechanisms and application. Commun. Agric. Appl. Biol. Sci., 68: 17-24.

Fernando WGD, Linderman RG (1994). Chemical control of stem and root rot of cowpea caused by *Phytophthora vignae*. Pl. Dis., 78: 967-971.

Girish K, Shankara Bhat S, Raveesha KA (2009a). *In vitro* screening systemic fungicides against *Phomopsis azadirachtae,* the incitant of die-back disease of neem. Arch. Phytopathol. Pl. Protect., 42: 256-264.

Girish K, Shankara Bhat S, Raveesha KA (2009b). *In vitro* evaluation of antagonistic microorganisms for the control of die-back of neem causal agent *Phomopsis azadirachtae.* J. Pl. Protect. Res., 49: 362-368.

International seed Testing Association (ISTA) (1993). International rules for seed testing. Seed Sci. Technol., 21: supplement.

Jacobsen BJ, Zidack NK, Larson BJ (2004). The Nature and application of biocontrol microbes: *Bacillus* spp. - The role of *Bacillus*-based biological control agents in integrated pest management systems: Plant diseases. Phytopathology, 94: 1272-1275.

Kiewnick S, Jacobsen BJ, Braun-Kiewnick A, Eckhoff JLA, Bergman JW (2001). Integrated control of *Rhizoctonia* crown and root rot of sugar beet with fungicides and antagonistic bacteria. Pl. Dis., 85: 718-722.

Ko WH, Kliejunas JT, Shimooka JT (1976). Effect of agar on inhibition of spore germination by chemicals. Phytopathology, 66: 363-366.

Krishna Kishore G, Pande S, Podile AR (2005a). Biological control of collar rot disease with broad-spectrum antifungal bacteria associated with groundnut. Can. J. Microbiol., 51: 123-132.

Krishna Kishore G, Pande S, Podile AR (2005b). Management of late leaf spot of groundnut (*Arachis hypogaea*) with chlorothalonil-tolerant isolates of *Pseudomonas aeruginosa*. Pl. Pathol., 54: 401-408.

Lavermicocca P, Valerio F, Evidente A, Lazzaroni S, Corsetti A, Gobbetti M (2000). Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol., 66: 4084-4090.

Lazarovits G, Nowak J (1997). Rhizobacteria for improvement of plant growth and establishment. Hort. Sci., 32: 188-192.

Maloy OC (1993). Plant disease control: Principles and practice. John Wiley & Sons. Inc., New York, U.S.A.

Mass JL, Galletta GJ (1997). Recent progress in strawberry disease research. Acta. Hort., 439: 769-780.

Maude RB (1996). Seed-borne diseases and their control: Principles and practice. CAB International, Cambridge, UK.

Meena NL, Shah R (2005). Efficacy of fungicides and phytoextracts against *Phomopsis citri* causing fruit rot in Mandarin orange var. Nagpur santra (*Citrus reticulata*). J. Mycol. Pl. Pathol., 35: 213-216.

Mundt CC, Cowger C, Garrett KA (2002). Relevance of integrated disease management to resistance durability. Euphytica, 124: 245-252.

Nautiyal CS (2001). Biocontrol of plant diseases for agricultural sustainability. In: Upadhyay RK, Mukerji KG, Champla BP (eds) Biocontrol potential and its Exploitation in Sustainable Agriculture, vol. 1: Crop diseases, Weeds and Nematodes, Kluwer Academic, New York, pp. 9-23.

Omar I, O'Neill TM, Rossall S (2006). Biological control of *Fusarium* crown and root rot of tomato with antagonistic bacteria and integrated control when combined with the fungicide carbendazim. Pl. Pathol., 55: 92–99.

Papavizas GC, Lewis JA (1988). The use of fungi in integrated control of plant disease. In: Burge MN (ed) Fungi in Biological Control Systems, Manchester University Press, Manchester, pp. 235-253.

Perkins JH, Patterson BR (1997). Pests, pesticides and the environment: a historical perspective on the prospects for pesticide reduction. In: Pimental D (ed) Techniques for Reducing Pesticide Use, John Wiley & Sons, Chichester, pp. 13-33.

Ponmurugan P, Baby UI, Gopi C (2006). Efficacy of certain fungicides against *Phomopsis theae* under *in vitro* conditions. Afr. J. Biotech., 5: 434-436.

Punam Singh, Khan SN, Singh P (1999). Assessment of seed-borne fungi of some forest trees and their management. Indian J. For., 22: 281-284.

Rathmell WG (1984). The discovery of new methods of chemical disease control: Current developments, future prospects and the role of biochemical and physiological research. In: Ingram DS, Williams PH (eds) Advances in Plant pathology, Academic Press, London.

Sateesh MK (1998). Microbiological investigations on die-back disease of neem (*Azadirachta indica* A. Juss.). PhD dissertation, University of Mysore, India.

Sateesh MK, Shankara Bhat S (1999). Detection of seed–borne *Phomopsis azadirachtae* and its transmission in *Azadirachta indica* (Neem). Seed Sci. Technol., 27: 753-759.

Sateesh MK, Shankara Bhat S, Devaki NS (1997). *Phomopsis azadirachtae sp. nov*. from India. Mycotaxon, 65: 517-520.

Shankara Bhat S, Sateesh MK, Devaki NS (1998). A new destructive disease of neem (*Azadirachta indica*) incited by *Phomopsis azadirachtae*. Curr. Sci., 74: 17-19.

Siddiqui IA, Ehteshamul-Haque S (2001). Suppression of the root rot–root knot disease complex by *Pseudomonas aeruginosa* in tomato: The influence of inoculum density, nematode populations, moisture and other plant-associated bacteria. Pl. Soil, 237: 81-89.

Singh DP, Maurya S, Prakash OM, Singh UP (2005). Phenolic composition and antifungal activity of culture filtrate of *Leptoxyphium axillatum*. Indian Phytopath., 58: 143-148.

Sunish Kumar R, Ayyadurai N, Pandiaraja P, Reddy AV, Venkateswarlu Y, Prakash O, Sakthivel N (2005). Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits. J. Appl. Microbiol., 98: 145-154.

Wang ZY, Liang XY, Shi D, Zhou HJ, Ye QM, Lin PL (1995). Screening of fungicides to *Phomopsis asparagi* Bubak and field control tests. Acta Agric. Zhejiang., 7: 457-463.

Zhang W, Watson AK (2000). Isolation and partial characterization of phytotoxins produced by *Exserohilum monoceras*, a potential bioherbicide for control of *Echinochloa* species. In: Spencer NR (ed) Proceedings of the X International Symposium on Biological Control of Weeds. Montana: Montana State University, pp. 125-130.