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Antifungal activity of secondary metabolites of *Pseudomonas fluorescens* isolates as a biocontrol agent of chocolate spot disease (*Botrytis fabae*) of faba bean in Ethiopia

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Pseudomonas fluorescens **isolates possess a variety of promising properties of antifungal activity of secondary metabolites which make it as a biocontrol agent. In the present study, 12 isolates of *P. fluorescens* were isolated from rhizospheric soil of faba bean crop evaluated for their antagonistic activity against chocolate spot disease (*Botrytis fabae*) of faba bean. *P. fluorescens* 10 (Pf 10) (88.1%) showed high antagonistic activity against *B. fabae*. All isolate of *P. fluorescens* were successfully employed in controlling chocolate spot disease of faba bean due to their antifungal metabolites. The antifungal compounds were extracted from all *P. fluorescens* isolates with equal volume of ethyl acetate, hexane and methanol. The antifungal compounds extracted with ethyl acetate, hexane and methanol from *P. fluorescens* 3 (Pf 3), *P. fluorescens* 8 (Pf 8), and *P. fluorescens* 3 (Pf 3), isolates at 0.1% concentration completely inhibited the mycelial growth of the pathogen respectively. Bio-primed faba bean seeds with isolates of *P. fluorescens* 9 (Pf 9) and *P. fluorescens* 10 (Pf 10) evaluated against *B. fabae in vivo* (pot culture) indicated the inhibitory effects to the pathogen and also showed the inducing properties to enhance the immune system of crop. Therefore, it can be concluded that the use of *P. fluorescens* 9 (Pf 9) and *P. fluorescens* 10 (Pf 10) of isolates could inhibit the mycelial growth and reduced the disease incidence, severity and infection processes of *B. fabae* and simultaneously increase the plant growth performance and yield of faba bean. These isolates can be used as potential biocontrol agents against *B. fabae* and also used as biofertilizers for the production of faba bean.**

Key words: Antifungal, Biocontrol, *Botrytis fabae*, Faba bean, *Pseudomonas fluorescens*.

INTRODUCTION

There is an urgent need to improve *Vicia faba* yield, since this plant remains an important part of the diet of both humans and domestic animals in many parts of the world, because of its high nutritive value in both energy

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and protein contents. Furthermore, faba bean supplies an important benefit to the crop by fixing atmospheric nitrogen in symbiosis with *Rhizobium leguminosarum* thus, reducing costs and minimizing impact on the environmental, which is why increasing the plant production is one of the major targets of the agricultural policy in several countries (Mahmoud et al., 2004).

However, this crop is subjected to many abiotic and biotic stresses that seriously compromise the final yields. Among the menacing biotic stresses, chocolate spot, caused by *Botrytis fabae*, is a worldwide disease capable of devastating the unprotected faba bean, result in harmful effects on growth, physiological activities and yield. Chocolate spot disease of faba bean is the most wide spread and destructive disease in Ethiopia with yield reductions of up to 61% on susceptible cultivars (Dereje and Beniwal, 1987). The problem of adequately protecting plants against the fungus by using fungicides has been complicated by development of fungicidal resistance and many chemicals traditionally used to control chocolate spot disease is less effective (Harrison, 1984), giving only partial disease control, high cost of their use and /or adverse effects on growth and productivity of faba bean as well as on the accompanying microflora (Khaled et al., 1995). Therefore, controlling *B. fabae* by biocontrol agents seemed to be better and preferred than the chemical control (Mahmoud et al., 2004). Bio-priming, a seed treatment system that integrates the biological and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008).

The diversity and beneficial activity of the plant-bacterial association and its understanding is important to sustain agro-ecosystems for sustainable crop production (Germida et al., 1998). *Pseudomonas fluorescens* is a gram-negative, rod-shaped, and non-pathogenic bacterium that is known to inhabit primarily the soil, plants, and water (Peix et al., 2009). It derives its name from its ability to produce fluorescent pigments under iron-limiting conditions (Baysse et al., 2003). These bacteria belong to soil microorganisms that develop one of the very important soil processes of denitrification. Biological control is a promising approach for management of plant diseases. Biocontrol agents of *P. fluorescens* are well characterized for their ability to produce antimicrobial compounds (Haas and De´fago, 2005).

The concept of biocontrol of plant diseases includes disease reduction or decrease in inoculum potential of a pathogen brought about directly or indirectly by other biological agencies (Johnson and Carl, 1972). Outside the host, the biocontrol agent may be antagonistic and thereby reduce the activity, efficiency and inoculum density of the pathogen through antibiosis, competition and predation/hyper parasitism. This leads to a reduction in inoculum potential of the pathogens (Baker, 1977). Bio-priming, a seed treatment system that integrates the biological and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008).

The addition of Carboxymethyl cellulose (CMC) or pectin to bio-primed seeds enhanced the antagonists' ability to grow and survive competitively. In addition, they had no effect on seed germination (Elzein et al., 2006). The present study was designed to isolate certain rhizospheric bacteria of *P. fluorescens* for their antagonistic and antifungal activity of secondary metabolites against chocolate spot disease (*B. fabae*) to reduce the disease incidence and severity in order to increase yield of faba bean.

MATERIALS AND METHODS

Soil sample collection and bacterial isolation

Rhizospheric soil samples were collected from fields growing faba bean (*Vicia faba* L.) from five localities area of Selale zones, Oromia Region, Ethiopia. The soil samples were brought to Mycology Laboratory, Addis Ababa University. 10 g of rhizosphere soil sample was suspended in 90 ml of sterile distilled water. Samples were serially diluted up to 10^5 to 10^6 and 0.1 ml of sample was spread on King's B medium plates (King et al., 1954). After incubation at 28°C for 48 h, the plates were exposed to UV light at 365 nm for few seconds and the colonies exhibiting the fluorescence were picked up and purified on King's B medium plates and 12 *P. fluorescens* isolates (Pf 1) were isolated and they were designed as Pf 1 up to Pf 12 for further studies.

Source of faba bean and chocolate spot disease

Faba bean seed used in the present work was obtained from Holleta Agriculture Research Centre, Ethiopia. Three varieties of faba bean seed were provided (such as: NC 58 susceptible variety, Moti moderate variety and ILB 938 relative resistant variety). One isolate of *Botrytis fabae* was obtained from Holleta Agricultural Research Centre, Ethiopia. This strain was isolated from the leaf of infected faba bean crops grown from Holleta areas.

In vitro evaluation of bacterial antagonit against the test pathogen

All *P. fluorescens* isolates were assessed for potential antagonistic activity against *B. fabae* on King's B agar using dual culture technique (Rangeshwaran and Prasad, 2000). An agar disc (4 mm) was cut from an actively growing (96 h) *B. fabae* culture and placed on the surface of fresh King's B agar medium at the center of the Petri plates. A loopful of actively growing *P. fluorescens* isolates was placed opposite to the fungal disc and the *P. fluorescens* isolates on the plate were streaked at four locations, approximately 3 cm from the center. Plates inoculated with pathogen and without bacteria were used as control. All *in vitro* tests of antagonism were performed triplicates, with new co-inoculations used each time. Plates were incubated at room temperature for 7 days. Degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control. The percentage of mycelial growth inhibition was calculated by the following equation (Riungu et al., 2008):

$$\text{Percent of Inhibition} = \frac{C - T)}{C} X\ 100$$

Where, C= Radial growth of fungus in control plates (mm) and T= radial growth of fungus on the plate inoculated with antagonist (mm).

Extraction of secondary metabolites of *P. fluorescens* isolates

All *P. fluorescens* isolates were grown in 100 ml of King's B media in 250 ml conical flask in orbital shaker at 28°C and 120 rpm, for 96 h. The culture was centrifuged at 10,000 rpm for 15 min to get the cell-free filtrate (Tripathi and Johri, 2002). Secondary metabolites were extracted by partitioning with organic solvents such as: ethyl acetate, hexane and methanol the three solvents (Tripathi and Johri, 2002). The antifungal compounds were extracted from cell-free broth with equal volume of ethyl acetate, hexane and methanol (1:1:1) and the extract was separated from the aqueous by using separating funnel and then evaporated in a rotary evaporator at 45, 60 and 65°C at 121 rpm to ensure complete solvent removal respectively. The extracted secondary metabolites without concentration were tested for their efficacy against pathogens by poison food technique (Nene and Thapliyal, 1973). The concentrations of extracted secondary metabolite (0.1%) (25 µm) were prepared and poured on King's B agar medium with mixed, before a 4 mm disc of *B. fabae* culture was inoculated at the center of each plate; three replications were maintained for each treatment and the Petri dishes were incubated at 28°C. King's B medium plates with only solvent served as control. After full growth of the control plate's size of colony, diameter measured in mm and percentage inhibition of mycelial growth was calculated using the formula (Mohana and Raveesha, 2007):

$$\text{Percent of Inhibition} = \frac{(C - T)}{C} X\ 100$$

Where, C = Average increase in mycelial growth in control plate and T=Average increase in mycelial growth in treatment plate.

Greenhouse experiment

Preparation of fungal inoculum

The inoculums of *B. fabae* were prepared from old culture grown on faba bean seed dextrose agar at 28°C. Conidia were harvested by scraping, transferred to sterilized distilled water and filtered through nylon mesh. Spore suspensions of *B. fabae* were adjusted to 2.5 × 10^5 spores mL^{-1} with sterile distilled water using a haemocytometer as described by Derckel et al. (1999).

Preparation of bacteria inoculum

P. fluorescens isolates were grown for 48 h in King's B (KB) broth medium, and then cells were harvested by centrifugation. Bacterial cell were resuspended in sterile distilled water and the concentration adjusted to 10^9-10^{10} cells/ml (El-Mougy and Abdel-Kader, 2008).

Bio-priming of faba bean seeds

Carboxymethyl cellulose (CMC) and pectin were used as adhesive polymers for the bio-priming process of three varieties of faba bean seeds with antagonistic biological agents. Two isolates of *P. fluorescens* were resuspended in sterile distilled water and the concentration adjusted to give 10^9-10^{10} cells/ml. 10 g of either CMC or pectin was resuspended in 1 L of *P. fluorescens* isolates suspensions. Seeds of faba bean (at the ratio of 500 g/L) were imbibed in each of the prepared priming solutions for 16 h (Jensen et al., 2004). The bio-primed seeds were then air-dried on filter paper for 1 h and stored in a refrigerator at 5°C until required. Another group of surface-sterilized faba bean seeds (70% ethanol for 2 min) was prepared as control treatments (El-Mougy and Abdel-Kader, 2008).

Pot experiments

The experiment were designed under greenhouse conditions in Ecology and Ecophysiology Greenhouse, Addis Ababa University in March 2012, using pots (21 cm) containing 4 kg of sterilized loamy clay soil. First, soils were infested with 20 ml of *B. fabae* spore suspension (2.5 × 10^5 spores/ml) by soil drenching (Haggag et al., 2006). The pots were irrigated for 7 days before bio-control agent inoculation. Afterward, four of the bio-primed faba bean seeds were sown in each pot. The experiment included the following treatments: 1) non-infested soil (control); 2) soil only treated with *B. fabae*; 3) *B. fabae* + *P. fluorescens* isolates (P f 9 and P f 10), separately. Pots were kept under greenhouse conditions until the end of the experiment (Abd-El-Khair et al., 2010).

Disease assessment

The disease incidence (DI) and disease severity (DS) of chocolate spot disease were recorded at the 50 and 70th day after planting of faba bean in *in vivo* condition in green house. The disease severity of chocolate spot disease was recorded at 50 and 70 days from sowing under natural infection by using the scale of Bernier et al. (1993) as follows:

1 = No disease symptoms or very small specks (highly resistance); 3 = few small discrete lesions (resistant); 5 = some coalesced lesion with some defoliation (moderate resistant); 7 = large coalesced sporulating lesions, 50% defoliation and some dead plant (Susceptible); 9= Extensive lesions on leaves, stems and pods, severe defoliation, heavy sporulation, stem girdling, blackening and death of more than 80% of plants (Highly susceptible).

Chocolate spot disease severity was assessed according to the scale of Bernier et al. (1984).

$$\text{Disease severity \%} = \frac{(nxv)}{9n} x100$$

Where, (n)= Number of plants in each category; (v)= Numerical values of symptoms category; (N)= Total number of plants; (9)= Maximum numerical value of symptom category.

The disease incidence of chocolate spot as a disease percentage was determined after 50 and 70 days from sowing the first treatment according to the following formula:

$$\text{Disease incidence} = \frac{\text{Number of infected leaf lets}}{\text{Total number of tested leaflets}} x100$$

The efficacy percentage (E %) of *P. fluorescens* (P f9 and P f10) in reducing disease severity percentage of faba bean was assessed according to the equation adapted by Rewal and Jhooty (1985) as follow:

$$\text{Percent of Inhibition} = \frac{\text{\% disease severity in control} - \text{\% disease severity treatment}}{\text{\% Disease severity in control}}$$

Figure 1. *Pseudomonas fluorescens* was isolated based on their pigment production under UV light at 365 nm.

Figure 2. *Pseudomonas fluorescens* isolates was confirmed again under UV light at 365 nm.

Pathogenicity test

Re- isolation of the pathogen

B. fabae was re-isolated from the leaf lesion of the control plants in the *in vivo* experiment. Leaf lesions were cut into pieces and surface sterilized with 70% ethanol for 2 min and rinsed three with sterile water in Petri plates. Pieces were dried with sterile filter paper, plated on faba bean seed extract dextrose agar (FDA) medium and incubated at 28°C for 7 days. The fungus was subculture for purification, and identification was done using microscopes observation of the spore morphology and comparison with the original culture.

Data analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD and mean ± SE. IBM SPSS 20 version statistical software package was used for statistical analysis of percentage inhibition and disease incidence and disease severity in each case.

RESULTS

Isolation of *P. fluorescens*

During this research work, 12 *P. fluorescens* were isolated from rhizospheric soil of healthy faba bean from five locality of Oromia region, Ethiopia, on King's B medium and observed under UV light at 365 nm for few seconds as shown in Figure 1. Then, it was purified again on same medium and observed under UV light as indicated in Figure 2. All the rhizospheric isolates were named as Pf 1 to Pf 12 as indicated in Table 1 (*P. fluorescens* isolate 1 = P f1, *P. fluorescens* isolate 2 = P f2, *P. fluorescens* isolate 3 = P f3, *P. fluorescens* isolate 4= P f4, *P. fluorescens* isolate 5 = P f5, *P. fluorescens* isolate 6 = P f6, *P. fluorescens* isolate 7 = P f7, *P. fluorescens* isolate 8 = P f8, *P. fluorescens* isolate 9 = P f9, *P. fluorescens* isolate 10 = P f10, *P. fluorescens* isolate 11 = P f11, *P. fluorescens* isolate 12 = P f12) and maintained on Nutrient Agar slants for further testing and biochemical production test. Spore morphology of *B. fabae* and the sporulation spores attachment to mycelia were observed under microscope by execution of slide culture as indicated in Figure 3.

In vitro evaluation of bacterial antagonistic activity against the test pathogen

The results of *in vitro* evaluation and testing of *P. fluorescens* isolates showering antagonistic activities towards *B. fabae* are shown in Table 1 and Figure 4. Inhibition was clearly discerned by very limited growth of fungal mycelium in the inhibition zone surrounding a bacterial colony. The antagonistic effects of *P. fluorescens* isolates against *B. fabae* were in the range of 84.1- 88.1%. Pf 10 gave the maximum inhibition about 88.1 %, followed by Pf 9 (88.0 %). Control plates were not treated with isolates of *P. fluorescens* completely covered by the *B. fabae*.

Antifungal activity of ethyl acetate extracts of secondary metabolites of *P. fluorescens* isolates against *B. fabae*

The results for all the *P. fluorescens* isolate are shown in Table 2. Ethyl acetate extracts of the isolate Pf 3 completely inhibited the growth of *B. fabae.* The maximum inhibition of mycelia growth of *B. fabae* was observed in extracts of Pf 9 (86.30%) and Pf 10 (85.20).

Antifungal activity of hexane extracts of secondary metabolites of *P. fluorescens* isolates against *B. fabae*

The result shown in Table 3 indicates that Pf 8 at 0.1% concentration totally inhibited the growth of mycelia and

Table 1. Effect of antagonistic activity of *P. fluorescens* isolates treatments against the leaner mycelial growth of *Botrytis fabae in vitro* tests.

***P. fluorescens* isolate**	**Antagonistic effect against *Botrytis fabae***	
	Mycelial diameter (cm) (Mean ± SD)	**Inhibition (%)**
Pf 1	2.30 ±0.26	87
Pf 2	2.63 ±0.32	85.4
Pf 3	2.43 ±0.21	86.5
Pf 4	2.86 ±0.12	84.1
Pf 5	2.73 ±0.31	84.8
Pf 6	2.63 ±0.49	85.4
Pf 7	2.23 ±0.25	87.6
Pf 8	2.40 ±0.36	86.7
Pf 9	2.20 ±0.00	88
Pf 10	2.13 ±0.15	88.1
Pf 11	2.46 ±0.50	86.3
Pf 12	2.80 ±0.20	84.8
Control	9.00 ±0.00	-

SD= standard deviation.

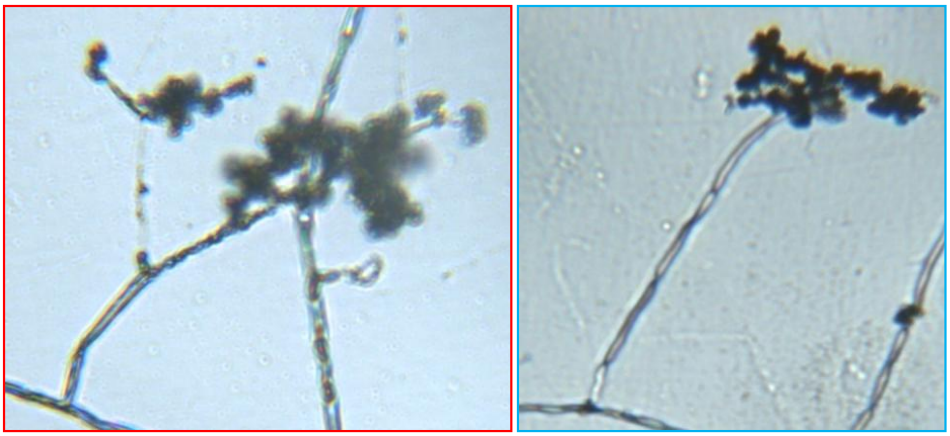

Figure 3. Conidiophore of *Botrytis fabae* (Bran|CHED dichotomously).

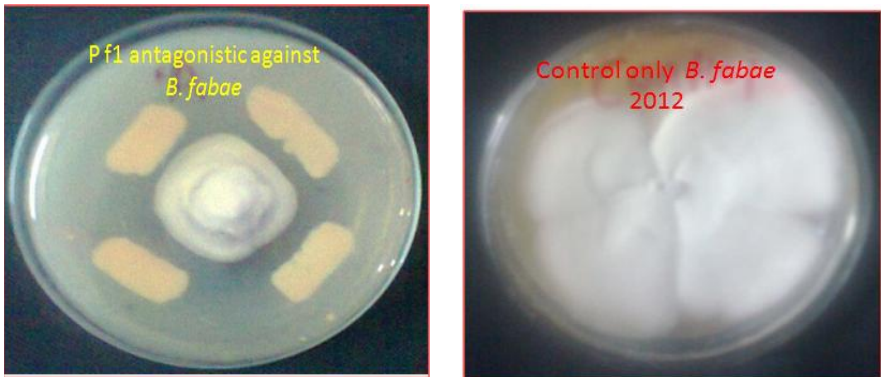

Figure 4. Dual culture of *Pseudomonas fluorescens* isolates with *B. fabae* on King's B medium.

Table 2. Percentage of inhibition of ethyl acetate extract of *Pseudomonas fluorescens* isolates metabolites at 0.1 % concentration against *B. fabae.*

Antifungal compounds of *P. fluorescens*	*B. fabae* isolates	
	MG (mm) (Mean ± SE)	INH %
Pf 1	15.33 ±1.45	82.96
Pf 2	18.00 ±2.08	80.00
Pf 3	No growth	100
Pf 4	14.67 ±1.45	83.70
Pf 5	16.33 ±0.88	81.85
Pf 6	14.67 ±1.76	83.70
Pf 7	15.33 ±1.45	82.96
Pf 8	14.83 ±0.60	83.52
Pf 9	12.33 ±0.88	86.30
Pf 10	13.33 ±0.88	85.20
Pf 11	15.67 ±1.20	82.60
Pf 12	16.67 ±1.20	81.48
Control	90.00± 0.00	-

MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean.

Table 3. Percent of inhibition of hexane extract of secondary metabolites of *Pseudomonas fluorescens* isolates at 0.1 % concentration against *B. fabae.*

Antifungal compounds of *P. fluorescens*	*B. fabae* isolates	
	MG (mm) (Mean ± SE)	INH %
Pf 1	18.67 ±2.33	79.26
Pf 2	17.00 ±1.73	81.11
Pf 3	18.67 ±1.20	79.26
Pf 4	20.67 ±1.45	77.04
Pf 5	22.67 ±0.88	74.81
Pf 6	21.33 ±1.86	76.30
Pf 7	23.00 ±1.53	74.44
Pf 8	No growth	100
Pf 9	13.00 ±0.58	85.60
Pf 10	16.00 ±2.52	82.22
Pf 11	20.33 ±1.20	77.41
Pf 12	18.00 ±1.53	80.00
Control	90.00 ±0.00	-

MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean.

the highest percent of inhibition on the growth of *B. fabae* was obtained with extracts of Pf 9 (85.60%) followed by P f10.

Antifungal activity of methanol crude extracts of secondary metabolites of *P. fluorescens* isolates against *B. fabae*

The effect of extracellular metabolites extracts of *P. fluorescens* isolates on the growth of *B. fabae* is shown in Table 4. The two effective extracts of Pf 3 and Pf 10 showed complete inhibition and highest percentage of inhibition of the mycelial growth of *B. fabae* respectively.

Pot experiments

Evaluation of bio-primed seeds of faba bean treatments with *P. fluorescens* isolates were the suppression of *B. fabae* disease incidence and severity investigated under artificial inoculation conditions (Table 5). The result of disease incidence, severity treatment and efficacy are shown in Table 5. Disease symptoms attributed to *B. fabae* were

Table 4. Percentage of inhibition of methanol extract of secondary metabolites of *Pseudomonas fluorescens* isolates at 0.1 % concentration against *B. fabae.*

Antifungal compounds of *P. fluorescens*	*B. fabae* isolates	
	MG (mm) (Mean ± SE)	INH %
Pf 1	19.33 ±1.20	78.52
Pf 2	20.00 ±1.53	77.78
Pf 3	No growth	100
Pf 4	18.00 ±0.58	80.00
Pf 5	17.33 ±1.67	80.74
Pf 6	22.00 ±.58	75.56
Pf 7	20.00 ±1.73	77.78
Pf 8	22.33 ±0.88	75.19
Pf 9	14.00 ±2.08	84.44
Pf 10	13.67 ±2.52	84.82
Pf 11	25.00 ±1.53	72.22
Pf 12	16.00 ±1.53	82.22
Control	90.00 ±0.00	-

MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean.

Table 5. Disease severity and incidence of chocolate spot disease (*Botrytis fabae*) on faba bean leaves treated with *P. fluorescens* isolate 9 and *P. fluorescens* isolate 10 under greenhouse condition.

Treatments and Controls	After 50 days			After 70 days		
	DS (%)	Efficacy(%)	DI (%)	DS (%)	Efficacy(%)	DI (%)
Pf 9 NC 58	11.11	40.01	25.00	11.11	57.14	41.67
Pf 9 Moti	3.70	66.70	8.33	11.11	40.01	33.33
Pf 9 ILB 938	3.70	66.70	8.33	3.70	80.02	16.67
Pf 10 NC 58	3.70	80.02	16.67	3.70	85.73	33.33
Pf10 Moti	3.70	66.70	8.33	3.70	80.02	16.67
Pf10 ILB 938	3.70	66.70	8.33	3.70	80.02	16.67
Negative Control NC 58	18.52	-	66.67	25.93	-	75.00
Negative Control Moti	11.11	-	33.33	18.52	-	66.67
Negative Control ILB 938	11.11	-	16.67	18.52	-	58.33
Positive Control NC 58	3.70	-	-	3.70	-	-
Positive Control Moti	3.70	-	-	3.70	-	-
Positive Control ILB 938	3.70	-	-	3.70	-	-

DS = disease severity, DI= disease incidence.

observed slightly on faba bean plants grown in soil artificially infested with bio-primed seeds of faba bean with two *P. fluorescens* isolates (Pf 9 and Pf 10) in pot experiment as compared with the control. Bio-primed seeds of faba bean Moti and ILB 938 with Pf 9 and Pf 10 showed lowest disease severity compared with the untreated plants after 50 days. In general, two isolates of *P. fluorescens* effectively reduced the disease on the susceptible (NC 58), moderately resistant (Moti) and relative resistant (ILB 938) whereas, disease incidence of bio-primed seeds of faba bean Moti and ILB 938 with P f9 and P f10 had the lowest compared with the untreated ones after 50 day. Disease severity was constantly delayed on NC 58, Moti and ILB 938 varieties during the observation period after 70 days. Disease incidence after 70 days, were lowest on 16.67% ILB 938 varieties with Pf 9 and Pf 10 compared with the untreated.

DISCUSSION

In the present study, *in vitro* evaluation of all *P. fluorescens* isolates treatments reduced the mycelial growth of *B. fabae* on King's B medium. It has been observed that the mycelial growth was reduced due to the production of secondary metabolites which inhibited growth of *B. fabae*. Similarly, *P. fluorescens* was shown

to effectively inhibit *R. solani* and *P. oryzae* by agar plate method (Rosales et al., 1995).

The present results of all *P. fluorescens* isolates showed the maximum inhibition (88.1%) of mycelial growth of *B. fabae. P. fluorescens* isolated from rhizosphere of organic farming area is effective against *Rhizoctonia solani* (Anitha and Das, 2011). *P. fluorescens* strain 003 was found to effectively inhibit (85%) the mycelial growth of *R. solani* (Reddy et al., 2007). *P. fluorescens* 003 was found to be highly effective in controlling *R. solani* with inhibition of 58% (Reddy et al., 2010). *P. flourescence* showed highest antifungal activity against *Penicillium italicum* (94%) and was moderately effective against *Aspergillus niger* (61%) (Mushtaq et al., 2010). Isolate of *P. fluorescens* on co-inoculation with fungal pathogens showed maximum inhibition for phytopathogens of *Collectotrichum gleosporioides* (58.3%), *Alternaria brassicola* (50%), *Alternaria brassiceae* (12.5%), *Alternaria alternate* (16.66%), *Fusarium oxysporum* (14.28%) and *R. solani* (50%) (Ramyasmruthi et al., 2012). *In vitro* evaluation of antifungal activity of ethyl acetate and methanol extracts of secondary metabolites of Pf 3 at 0.1% concentration revealed that they completely inhibited the mycelial growth test pathogen (*B. fabae*) compared to hexane solvents, suggesting that the antifungal compound are completely extracted with ethyl acetate, methanol and slightly extracted with hexane. Similarly, Reddy et al. (2007) reported that the crude compounds from *P. fluorescens* isolates metabolites completely inhibited the growth of *Magnaporthe grisea, Dreschelaria oryzae*, *R. solani* and *Sarocladium oryzae* at 5%. The antifungal activity of the three solvent extracts of secondary metabolites of Pf 1, Pf 2, Pf 4, Pf 5, Pf 6, Pf 7, Pf 9, Pf 10 and Pf 11 showed that ethyl acetate extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with ethyl acetate than methanol and hexane. Similarly, the metabolite extracted from *P. fluorescens* with ethyl acetate was effectively inhibited (89-90%). *P. oryzae* and *R. solani* were tested at 5% concentration (Battu and Reddy, 2009). The culture filtrates obtained from *P. fluorescens* showed the inhibition of 55.2% against *Stenocarpella maydis* (Petatán-Sagahón et al., 2011). It has been observed that the filtrates obtained in logarithmic phase from the *P. fluorescens* 16 inhibited 54% of the growth of *Stenocarpella maydis* (Petatán-Sagahón et al., 2011).

Petatán-Sagahón et al. (2011) observed that the culture filtrates obtained from *Pseudomonas* spp. showed a low inhibition (5.0%) against *Stenocarpella maydis*. Maleki et al. (2010) observed that the antifungal activity of *P. fluorescence* CV6 showed higher mycelial inhibition against *Colletotrichum gloeosporioides.* The maximum inhibition of conidial germination of *Fusarium oxysporum* was brought out by 2% *P. fluorescens* (83.15 %) and the inhibition of radial mycelial growth of pathogen was effected by 2% concentration of culture filtrate of *P. fluorescens* (60.0 %) (Rajeswari and Kannabiran, 2011).

In vitro evaluation of antifungal activity of hexane extracts of secondary metabolites of Pf 8 revealed that it completely inhibited the tested pathogen *B. fabae* compared to hexane and methanol solvents, suggesting that the antifungal compound are completely extracted with hexane and slightly with ethyl acetate and methanol. *In vitro* evaluation of *Pseudomonas* spp showed antifungal activity against *Verticillium dahliae var. longisporum* as potential biocontrol agents (Berg et al., 1998).

Bioassay activity of the three solvent extracts of secondary metabolites of Pf 12 showed that methanol extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with methanol than ethyl acetate and hexane. Maleki et al. (2010) had reported that antifungal activity of *P. fluorescence CV6* showed the highest mycelial growth of inhibition against *Magnaporthe grisea*.

Application of bio-primed faba bean seed (Moti or ILB 938) with Pf 9 gave the maximum reduction of chocolate spot severity at 50 days after planting of faba bean, but at 70 days, the highest reduction was recorded on ILB 938 variety whereas bio-primed faba bean seed (NC 58) with Pf 10 gave the highest reduction of chocolate spot severity at 50 and 70 days after planting of faba bean. Generally, it may be related to the ability of Pf 9 and Pf 10 to stimulate the phenol and flavonoids in faba bean plant associated with increased protection and acquired immune system against chocolate spot disease (*B. fabae*) in the crop. Data clearly indicated that in untreated plants, chocolate spot infection gradually increased on leaves during growth periods and great differences were obtained among treatments of Pf 9 and Pf 10 and untreated control.

It has been showed that the bio-priming of seeds with bacterial antagonists increases the population load of the antagonist 10-fold on the seeds and thus protected the rhizosphere from the invasion of plant pathogens (Callan et al., 1990). Furthermore, the use of bio-priming seeds could be considered a safe, cheap and easily applied biocontrol method to be used against soil borne plant pathogens and physiological aspects of disease control which involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008). *P. fluorescens* strain possessing multiple mechanisms of broad spectrum antagonism and PGP activities can be explored as one among the best biocontrol agent (Ramyasmruthi et al., 2012). Maleki et al. (2010) reported that *P. fluorescence* CV6 had a broad spectrum antifungal activity against phytopathogens that can be used as an effective biological control candidate against devastating fungal pathogens that attack various plant crops. Tesfaye and Kapoor (2004 and 2007) also observed that *Trichoderma* and *Gliocladium* have greatest potential for the control of *Botrytis* corm rot (*Botrytis gladiolorum*) of *Gladiolus in vitro* and *in vivo* conditions.

To conclude, application of fungicides for disease control are largely affecting human health, normal flora and fauna, soil microorganisms and environment and also lead to the pathogenic fungi becoming very fast resistant to fungicides. For this reason, seed inoculation with *P. fluorescens* isolates as a bio-primed seed that showed antagonistic activities against *B. fabae* is an acceptable alternative to chemical fungicides application. Based on the present studies, *P. fluorescens* isolates under investigation possess a variety of promising properties which make them better biocontrol agents that are capable of producing antifungal substances and subsequent enhancement of yield of faba bean crop. The uses of *P. fluorescens* isolates Pf 9 and Pf 10 as bio-primed faba bean seed are an effective strategy for management of chocolate spot disease as well as reducing disease severity and incidence in faba bean in green house during pathogenicity test. The result of this study indicate that *P. fluorescens*, Pf 9 and *P. fluorescens* Pf 9 isolates have great contribution for control of chocolate spot disease (*B. fabae*) of faba bean *in vitro* and *in vivo* conditions.

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