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EVALUATION OF PATHOGENIC ISOLATES IN ETHIOPIA FOR THE CONTROL OF CHOCOLATE SPOT IN FABA BEAN

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ABSTRACT

Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes in Ethiopia and is now cultivated on large areas in many countries. Production of the crop is, however, constrained by several disease infections including fungal diseases. The objectives of this work was to find out natural prevalence of *Bacillus* isolates on faba bean leaves in Amhara Regional state of Ethiopia; and to identify potential biocontrol agents for the management of chocolate spot (*Botrytis fabae).* Thirty eight distinct groups of isolates of *Bacillus* species out of a total 110 isolates obtained from 12 districts of north-west Ethiopia were found to occur on faba bean leaves. They differed in morphological and cultural characteristics. Thirty isolates of *Bacillus* spp. were tested for their effects on *Botrytis fabae* pathogen by dual culture technique on potato dextrose agar. Sixteen isolates produced 5 mm or higher inhibition zone and out of these isolate 43y and 56y were the most effective having inhibition zone of 8 and 7 mm, respectively. Isolates reduced the growth of the pathogen colony in dual culture by 23- 64%. The highest reduction was caused by isolate 115y (64%), followed by 114-3y (52%) and 29y and 67y (50% each). Isolates of *Bacillus* were placed on actively growing colonies of *B. fabae* and were found to cause lysis of *B. fabae* mycelium. Eleven isolates caused 8 mm or higher lytic area of mycelium. Maximum lysis of mycelium was caused by isolates 111-1y (16.8 mm) and 116y (11.3 mm), and they were confirmed from CABI Global Plant Clinic as *Bacillus* and not pathogenic to plants and animals. Further evaluation of promising antagonistic isolates by the detached leaf technique showed that most of them reduced the disease development on leaves. However, the degree of disease reduction varied with cultivar. Isolates 108-2y, 20-2y, 47-2y and 36-1y proved most effective in retarding disease development on two susceptible and one tolerant cultivar and can be further explored for commercial use for management of chocolate spot disease of faba bean.

Key Words: *Bacillus* spp., Biocontrol, *Botrytis fabae*, Ethiopia

RÉSUMÉ

Le haricot faba (*Vicia faba* L.) est l'un des premiers domestiqués légumineuses alimentaires en Ethiopie et en est maintenant cultivé sur de grandes superficies dans de nombreux pays. La production de plant est toutefois limitée par des infections de plusieurs maladies don't les maladies fongiques. Les objectifs de ce travail étaient de découvrir la prévalence naturelle des isolats de *Bacillus* sur des feuilles de haricot faba dans la régions d'Amhara en Éthiopie; et d'identifier d'éventuels agentd de lutte biologique pour la gestion de taches chocolats (*Botrytis fabae)*. Trente huit groupes distincts d'isolats d'espèces de *Bacillus* sur un total de 110 isolats obtenus à partir de 12 districts du Nord-Ouest de l'Éthiopie avaient été trouvés présents sur des feuilles de haricot faba. Ils différaient par les caractéristiques morphologiques et culturales. Trente isolats de *Bacillus* spp. avaient été testés pour leurs effets sur le pathogène *Botrytis fabae* par la technique de double culture sur gélose de dextrose à base de pomme de terre. Seize isolats avaient produit 5 mm ou bien une zone d'inhibition supérieure. De ces isolats, 43y et 56y étaient les plus efficaces car ayant respectivement des zones d'inhibition de 8 et 7 mm. Les isolats réduisaient de 23-64 % la croissance de la colonie des pathogènes sur la double culture. La réduction la plus élevée avait été causée par l'isolat 115y (64 %), suivie de 114-3y (52 %) et 29y et 67y (50 % chacun). Des isolats du

Bacillus avaient été placés sur les colonies de *B. fabae* à croissance rapide et s'étaient révélés comme étant la cause de la lyse du mycélium de *B. fabae.* Onze isolats causaient 8 mm ou plus de zone lytique du mycélium. La lyse maximale du mycélium avait été causée par les isolats 111-1y (16,8 mm) et 116y (11,3 mm), et ils étaient confirmés provenir de CABI Global Plant Clinic tel que *Bacillus* et ceux non pathogénique aux végétaux et animaux. Une évaluation prometteuse et plus approfondie des isolats antagonistes par la technique de feuilles détachées avait montré que la plupart d'entre eux réduisaient le développement de la maladie sur les feuilles. Cependant, le degré de réduction de la maladie variait avec le cultivar. Les isolats 108-2y, 20-2y, 47-2y et 36-1y s'étaient avérés plus efficaces en retardant le développement de la maladie sur deux cultivars sensibles et un seul tolérant; et peuvent être explorées davantage pour l'usage commercial dans la gestion de la maladie de tache chocolat du haricot faba.

Mots Clés: *Bacillus* spp., Biocontrol, *Botrytis fabae,* Éthiopie

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes and is now cultivated on large areas in many countries due to its high nutritive value in terms of energy and protein contents (24-30%). China leads in production, followed by Ethiopia, Egypt, Italy and Morocco (Bouhassan *et al*., 2004). The crop occupies the largest area among the pulses in Ethiopia. It is grown on 370,000 hectares with an annual production of about 450,000 tonnes (ICARDA, 2006). Even though Ethiopia is the world's second largest producer of faba bean, its share is only 6.96% of world production and 40.5% of Africa (Asfaw e*t al*., 1994).

The production of faba bean is constrained by several yield limiting factors, of which diseases are the main factors (Samia, 2006). Chocolate disease of faba bean caused by *Botrytis fabae* Sard. is the most widespread and destructive disease in Ethiopia with yield reductions of up to 61% on susceptible cultivars (Dereje and Beniwal, 1987). In some countries, chocolate spot is caused by *Botrytis cinerea*, but in Ethiopia, only *B. fabae* is known to cause the disease. Chocolate spot initially appears as reddish brown spots on leaves, which enlarge and even merge and subsequently lead to sever premature defoliation. Under favourable conditions, it appears on stems, flowers and pods, and this directly affect seed production.

A number of management options have been developed in other countries to minimise the effects of chocolate spot on faba bean yield. These include use of resistant/tolerant varieties; use of cultural practices such as crop rotation, crop residue management, adjusting planting dates, and fungicide application (Bretag and Raynes, 2004; Hawthorne, 2004). In Ethiopia, growing of moderately resistant varieties, application of chlorothalonil or mancozeb and late planting have been recommended (Dereje, 1999; Sahile, 2008a). Large scale extensive application of chemical fungicides is neither feasible for resource poor farmers, nor environmentally desirable and safe. Moreover, the genetic base for resistance against chocolate spot is narrow in faba bean and highly resistant varieties are not available. Therefore, chemical control combined with cultural practices and moderately resistant cultivars is widely used to overcome the problem in other countries (Hawthorne, 2004). However, resistance to chemical fungicides has also been detected in *B. cinerea* and *B. fabae* (Parry, 1990; Maggie *et al.*, 2006).

Biological control with microbes is another feasible alternative option to the use of synthetic chemicals and is now becoming a critically needed component of integrated disease management (Obagwu and Korusten, 2003; University of Sydeney, 2003). A number of fungi and bacteria are known to be very effective against soil borne diseases. Research has shown that foliar diseases can also be managed effectively through microorganisms. Among these, *Bacillus* spp. have been found to be very important.

Bacilli generally have simple nutritional requirements, are able to colonise dry surfaces for long period of time, they rapidly utilise many of the available nutrients and can sustain many of the environmental hazards. Several potent strains from different species of *Bacillus* have been tested on a wide variety of plant species for ability to control several diseases and some have

been commercialised already. Among the different bacteria, *Bacillus* and *Pseudomonas* have been reported to have greatest potential to control *Botrytis* diseases (Elad and Stewart, 2004). Strain BS 153 of *Bacillus* has been reported to prevent chocolate spot symptoms on faba beans both in greenhouse and field studies (Sherga, 1997; Upadhyay *et al.*, 2000).

The objectives of this work was to find out natural prevalence of *Bacillus* isolates on faba bean leaves in Amhara Regional State of Ethiopia; and to identify potential biocontrol agents for the management of chocolate spot.

MATERIALS AND METHODS

Bacteria sample collection and isolation. Samples of healthy looking faba bean leaves were collected in 2004 and 2005 cropping season from farmers' fields in 12 districts of Amhara Regional State in Ethiopia, located between longitude 36^0-40^0 W to E, and latitudes 11^0 - 13^0 45'SW to N. One cm^2 pieces of tissues from the collected leaves were cut and plated on potato dextrose agar (PDA). The plates were incubated at 20-22^0 C and developing young colonies of bacteria were transferred to fresh PDA plates and purified. PDA was selected as growth medium, so that their subsequent *in vitro* tests with *B. fabae* could be made on the same medium. Isolates grown in pure culture were coded and transferred to screw-capped culture bottles containing faba bean seed extract dextrose agar. This followed the procedure of 200 g of faba bean seeds added in 1 lit of distilled water and autoclaved for 30 minutes. The extract of autoclaved beans was passed through a 2 mm sieve, mixed with 18 g of agar, heated and dissolved. Then 20 g of dextrose was added prior to autoclaving for 20 minutes. It was cooled and poured into petri-dishes and potato dextrose agar. Isolates were stored at 4°C until further use.

Identification of *Bacilli*. All bacterial isolates were Gram stained and those having Gram positive reactions were tested for catalase reaction (10% hydrogen peroxide was poured on the bacterial lawns to determine whether catalase was present). All catalase positive, rod-shaped and endospore forming bacteria (*Bacillus*) were selected for further studies. From these, human pathogenic *Bacillus* isolates were excluded using egg yolk mannitol bromothymol blue agar. The remaining 110 *Bacillus* isolates were maintained at 4 °C on slants of oxoid nutrient agar.

Growth rates *Bacillus* spp. isolates were compared on PDA at 21±1 °C so as to find out relatively fast growing isolates. On the basis of colony colour, margins, appearance and growth rate, the isolates were grouped into 38 groups of 110 isolates. From each group, one representative isolate was taken for antagonistic evaluation.

EVALUATION OF *BACILLUS* ISOLATES FOR BIOCONTROL POTENTIAL

***In vitro* testing of *Bacillus* isolates against *B. fabae*.** The *Bacillus* isolates from the leaves were tested for antagonistic potential on PDA in 9 cm diameter petri-dishes, against aggressive isolate (BK-117) of *B. fabae* obtained from Kutaber district.

Testing for antibiotic activity of *Bacillus* isolates. All isolates of *Bacillus* spp. were tested for antibiotic production and inhibition effects on the test pathogen isolate by dual culture methods. Five mm diameter disc of five-day-old culture of the pathogen were placed near the periphery of petri-dish and a loopful of *Bacillus* spp. isolate was placed at the opposite periphery of the plate containing PDA (Dhingra and Sinclair, 1986). The inoculated plates were incubated along with culture plates with no antagonistic isolate at 21±1 °C for 5-10 days.

The experiment was replicated three times in completely randomised design. Data on growth inhibition zone and colony diameter of pathogen and *Bacillus* species were recorded for each plate and inhibition of mycelial growth of the pathogen over control without *Bacillus* was calculated.

Testing for lytic activity of *Bacillus* isolates. Five millimeter discs of pathogen mycelium were placed on 15 ml of PDA in petri-plates and incubated at 21±1^{0}C. After three days of mycelial growth, one loopful of the isolate of *Bacillus* sp grown for 48-72 hr, was placed on the actively growing colony of the pathogen and incubated at 21±1 °C for 15 days. Lysis of pathogen

mycelium was examined periodically under a stereomicroscope (40 x magnification) and the width of lysed mycelia around the colony of the bacteria was measured. The experiment was conducted in completely randomised design and replicated three times. Culture plates having *B. fabae* but no *Bacillus* spp. isolate were used as control.

Isolates of *Bacillus* spp. that showed promising antagonistic activity under dual culture and lysis test were identified from CAB International Global Plant Clinic (UK).

Antagonistic activity i*n vivo*. Twenty isolates which showed substantial inhibition/lysis and having higher growth rate were further evaluated for antagonistic potential by detached leaves technique (Paul *et al.*, 1995). This was done on three faba bean cultivars namely, CS20DK (tolerant), FB-EH0013-18 (susceptible) and one local susceptible check. Leaves of the same age group of the three cultivars were taken and surface sterilised with 70% alcohol. They were placed on glass rods kept on sterile and moistened blotters in petri-plates. A conidial suspension of *B. fabae* (2.5×10^5 spores ml^{-1}) was prepared according to Mohammed *et al*. (1994). A drop of the conidial suspension (1 ml) was placed near the midrib proper. The petri-dishes having moist filter paper below were covered with the petri-dish lid and incubated for 24 hours at 21±1°C.

After 24 hour, a loopful of *Bacillus* isolate was added to the midrib proper, where the drop of pathogen suspension was placed and incubated at 21±1°C. A control was prepared with distilled water and another control with only pathogen suspension.

The experiment was arranged in a randomised complete block design (RCBD) with three replications. Severity rating of chocolate spot on detached leaves was assessed at 48, 72, 96 and 120 hr after inoculation using a 1-5 scale (ICARDA, 1986).

All analyses of variance (ANOVA) on inhibition zone, lysis effect and growth rates were carried out using SAS statistical analysis package (Ver. 8). Mean separation was done using LSD test at 5% probability level.

RESULTS

Prevalence of *Bacillus* species on faba bean. Table 1 shows that *Bacillus* species were widely prevalent on faba bean leaves in north-west Ethiopia. *Bacillus* isolates were obtained from 110 samples of faba bean leaves from 12 districts surveyed (Fig. 1). On the basis of colony

TABLE 1. Number of effective isolates and their distribution of *Bacillus* species isolates from faba bean leaves in different districts of north-western Ethiopia during 2004 and 2005 cropping seasons

Districts	Altitude range (m.a.s.l.)	Effective isolates recovered	Effective isolates code recovered	Potential *Bacillus* isolates within each district (%)
Yilmana-Densa	1980-2405	4	4y, 6By, 6-2y, 8y	10.5
Hulet-Eju-Ensae	2275-2670	4	11-2y, 20-1y, 20-2y, 21y	10.5
Gonder-Zuria	1969-2463	3	28y, 29y, 30-3y	7.9
Wogera	2650-2943	5	34y, 34-1y, 34-3y, 36-1y, 40b	13.2
Debark	2740-3053	5	41 y, 43 y, 45 y, 47-2 y, 49y	13.2
Farta	1975-3000	1	56 y	2.6
Lay-Gaynt	2794-3184	3	61 y, 64 y, 67 y,	7.9
Meket	2779-3319	2	74 y, 76 y	5.3
Gubalafto-Woldia	1900-3033	-	-	0
Ambasel-Tehulederae	1908-2196	1	108-2 y	2.6
Kutaber	2144-3250	9	111-1 y, 112By, 113 y, 114-2 y, 114-3y, 115y, 116y, 120-2y, 126y	23.7
Desei-Zuria	2055-3138	1	134-1y	2.6

- No isolate obtained

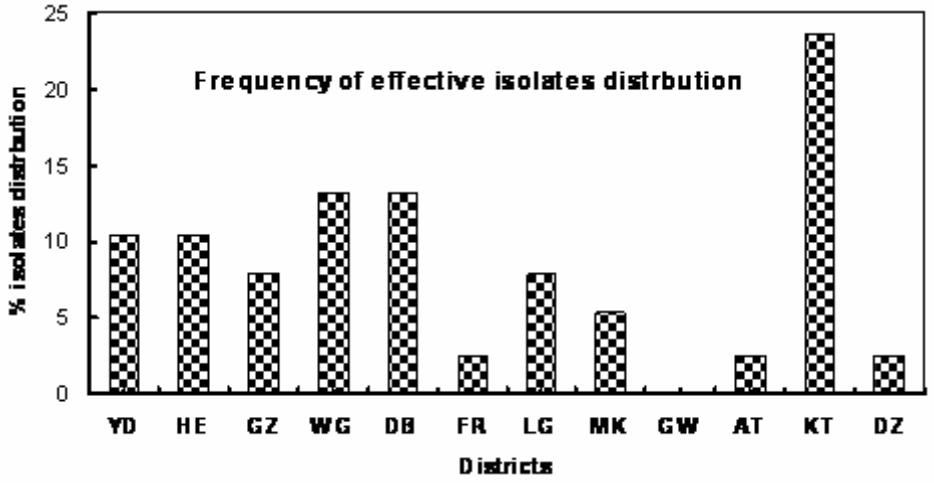

Figure 1. Percentage of effective isolates and their distribution of *Bacillus* spp. from faba bean leaves in different districts (YD=Yilmana-Densa, HE=Hulet-Eju-Ensae, GZ= Gonder-Zuria, WG=Wogera, DB=Debark, FR=Farta, LG=Lay-Gaynt, MK=Meket, GW= Gubalafto-Woldia, AT=Ambasel-Tehulederae, KT= Kutaber, and DZ=Desei-Zuria) of northwestern Ethiopia during 2004 and 2005 cropping seasons.

characters and growth rates, they were grouped into 38 distinct groups. Maximum number (9) isolates was obtained from Kutaber district followed by Debark and Wogera (5 each), and Yilmana densa and Hulet-Eju-Ensae (4 each). No *Bacillus* isolate was obtained from Gubalafto-Woldia, while from Farta , Ambasel-Tehulederae and Desei-Zuria only one isolate was obtained from each (Table 1).

There appeared was hardly any correlation between occurrence of *Bacilli* and altitude. All the 38 *Bacillus* isolates showed white creamy colour on both front and reverse sides of the plate. Most of the isolates started marked growth within 48 hr of incubation and relatively fast growth of most of the isolates was observed between 72-120 hr of incubation period (Table 2). Eleven isolates out of 38 showed slight reduction in growth rate after 96 hr. Among all isolates, 115y from Kutaber district showed highest growth rate of 7.2 mm/day consistently. Isolates 20-2y, 21y, 28y, 29y, 34-3y, 40y, 49y, 67y, 114-2y, 114-3y, 120-2y and 134-1y had more than 5 mm growth/day. Slowest growing isolates were 56y from Farta, and 64y from Lay Gaynt, growing <3 mm day^{-1}. Other isolates were intermediate in growth.

Antagonistic potential of *Bacillus* isolates against *B. fabae in vitro.* Significant differences ($P<0.05$) were observed among isolates in formation of inhibition zones in dual culture (Table 3). The highest inhibition zone (8 mm) was observed from isolates 43y (8 mm) and 56y (7 mm) obtained from Debark and Farta districts, respectively. Sixteen isolates produced 5 mm or higher inhibition zones. The lowest inhibition zone (3 mm) was observed with isolates 8y, 28y, 30-3y, 34-3y, 76y, 111-1y, 114-2y, and 116y, obtained from Yilmana Densa, Gondar Zuria, Wogera, Meket and Kutaber districts.

All tested isolates produced inhibition zones and also reduced growth of *B. fabae* in the dual culture (Table 3). Maximum reduction in growth rate of pathogen was observed with 115y, which restricted the growth to 16.2 mm compared to 45 mm in the control. Sixteen isolates restricted growth to less than 25 mm and showed 45-64% efficacy. In most cases, the antagonist *Bacillus* spp. was able to inhibit mycelia growth as well as affect the formation of conidia. In some cases, complete failure of conidial formation was observed.

Significant differences ($P<0.05$) were observed among *Bacillus* isolates in causing lysis of pathogen mycelium, when inoculated on actively growing colony of *B. fabae* (Table 3). The lysed areas formed by the different antagonistic isolates ranged from 5 to 16.8 mm.

TABLE 2. Colony growth rate of different antagonistic *Bacillus* isolates collected from faba bean crops of north-western Ethiopia

District	Isolate	Altitude (m. a.s.l)	Colony growth rate (mm/day)			
			48 hr	72 hr	96hr	120hr
Yilmana Densa	4y	2390	1.45	4.2	4.2	4.2
Yilmana Densa	6By	2465	3.8	3.8	3.8	3.8
Yilmana Densa	6-2y	2465	5.45	5.5	5.5	5.4
Yilmana Densa	8y	2386	4.95	4.9	4.9	5
Hulet Eju Ensae	11-2y	2406	3.6	3.6	3.6	3.6
Hulet Eju Ensae	20-1y	2597	4.55	4.5	4.5	4.6
Hulet Eju Ensae	20-2y	2597	5.35	5.3	5.3	5.4
Hulet Eju Ensae	21y	2576	5.2	5.2	5.2	5.2
Gonder Zuria	28y	2037	5.45	5.5	5.5	5.4
Gonder Zuria	29y	2075	5.6	5.6	5.6	5.6
Gonder Zuria	30-3y	2241	3.8	3.8	3.8	3.8
Wogera	34y	2064	5.05	5.1	5.1	5
Wogera	34-1y	2064	4.65	4.7	4.7	4.6
Wogera	34-3y	2064	5.2	5.2	5.2	5.2
Wogera	36-1y	2118	4.25	4.3	4.3	4.2
Wogera	40y	2689	5.2	5.2	5.2	5.2
Debark	41y	2835	2.9	2.8	3.6	2.8
Debark	43y	2920	3.6	3.6	3.6	3.6
Debark	45y	3006	4.55	4.5	4.5	4.6
Debark	47-2y	2852	4.2	4.2	4.2	4.2
Debark	49y	3122	5.45	5.5	5.5	5.4
Farta	56y	2851	2.8	2.8	2.8	2.8
Lay Gaynt	61y	2651	3.4	3.4	3.4	3.4
Lay Gaynt	64y	2173	2.6	2.6	2.6	2.6
Lay Gaynt	67y	2663	5.6	5.6	5.6	5.6
Meket	74y	3161	4.8	4.8	4.8	4.8
Meket	76y	3249	4.95	4.9	4.9	5
Ambasel Tehulderae	108-2y	2003	3.7	3.7	3.7	3.7
Kutaber	111-1y	2118	5.05	5.1	5.1	5
Kutaber	112By	2131	4.8	4.8	4.8	4.8
Kutaber	113y	2213	3.4	3.4	3.4	3.4
Kutaber	114-2y	2264	5.6	5.6	5.6	5.6
Kutaber	114-3y	2264	5.85	5.9	5.9	5.8
Kutaber	115y	2297	7.2	7.2	7.2	7.2
Kutaber	116y	2365	4.95	4.9	4.9	5
Kutaber	120-2y	2505	5.45	5.5	5.5	5.4
Dessei Zuria	126y	2336	3.4	3.4	3.4	3.4
Dessei Zuria	134-1y	2679	5.45	5.5	5.5	5.4

The highest lysed area was observed with *Bacillus* isolates 111-1y (16.8 mm), followed by 116y (11.3 mm) obtained from Kutaber district, while isolates 20-1y, 21y and 34y from Hulet-Eju-Eensae and Wogera districts showed lowest lysis (5 mm each). The lysed area changed the colour from gray to brown around the antagonists in the petri-plates.

Evaluation of promising isolates *in vivo.* Isolates of *Bacillus* which were moderate to fast growing, exhibited substantial inhibition zones, and curtailed pathogen growth and caused lysis were further evaluated for antagonistic potential in reducing disease development on detached faba bean leaves of three cultivars. Twenty isolates were tested against virulent isolate of *B. fabae*

TABLE 3. Performance of *Bacillus* species inhibition and lysis effect against *Botrytis fabae* on PDA

Isolates code	*Bacillus* colony reaction			*Bacillus* colony growth of 96 hr (in mm)	Mean radial growth of *B. fabae* at 96 hr (in mm)
	Inhibition	Lyses	Efficacy (%)		
4y	4	7.8	25.1	10.3	33.7
6By	6	8.5	33.8	14.2	29.8
6-2y	4	9.0	48.6	20.9	23.1
8y	3	6.3	43.8	18.7	25.3
11-2y	4	7.8	32	13.4	30.6
20-1y	5	5.0	40.3	17.1	26.9
20-2y	6	7.0	47.4	20.3	23.7
21y	4	5.0	46.2	19.8	24.2
28y	3	7.0	48.6	20.9	23.1
29y	4	7.8	49.8	21.4	22.6
30-3y	3	8.5	33.8	14.2	29.8
34y	5	5.0	45.1	19.3	24.7
34-1y	5	6.3	41.5	17.7	26.3
34-3y	3	6.5	46.2	19.8	24.2
36-1y	4	7.8	37.9	16.1	27.9
40y	4	6.3	46.2	19.8	24.2
41y	5	7.5	27.1	11.2	32.8
43y	8	7.8	32	13.4	30.6
45y	4	9.5	40.3	17.1	26.9
47-2y	5	8.5	37.3	15.8	28.2
49y	4	7.5	46.2	20.9	23.1
56y	7	7.8	24.9	10.2	33.8
61y	6	7.5	30.2	12.6	31.4
64y	5	7.0	23.1	9.4	34.6
67y	6	7.5	49.8	21.4	22.6
74y	4	8.5	42.7	18.2	25.8
76y	3	7.0	43.8	18.7	25.3
108-2y	4	7.5	32.9	13.8	30.2
111-1y	3	16.8	45.1	19.3	24.7
112By	5	8.0	42.7	18.2	25.8
113y	5	7.5	30.2	12.6	31.4
114-2y	3	9.0	49.8	21.4	22.6
114-3y	4	7.0	52.2	22.5	21.5
115y	4	6.5	64	27.8	16.2
116y	3	11.3	43.8	18.7	25.3
120-2y	5	9.5	46.2	20.9	23.1
126y	4	6.3	30.2	12.6	31.4
134-1y	6	7.5	46.2	20.9	23.1
Mean	4.5	7.7	40.7	17.7	26.6
Control					4.5
LSD (5%)	1.2	1.75		1.7	

and significant ($P<0.05$) differences occurred between *Bacillus* isolates on local cultivar in reducing the disease. Twelve isolates of *Bacillus* reduced the chocolate spot development within 5 days of inoculation on a susceptible cultivar EH0013-18; 11 isolates on tolerant cultivar (CS20DK); and 10 isolates on susceptible local check out of the total 20 evaluated. Out of these, isolates 41y, 112By, 30-3y and 108-2y proved more effective on EH0013-18, 61y and 6By on

TABLE 4. *In vitro* effect of *Bacillus* isolate *on* chocolate spot severity using detached leaf

Isolate code	Faba bean variety											
	FB-EH0013-18				CS20DK				Local check			
	48 hr	72 hr	96 hr	120 hr	48 hr	72 hr	96 hr	120 hr	48 hr	72 hr	96 hr	120 hr
47-2y	1.0	1.0	1.5	1.5	1.0	1.0	1.0	1.5	1.0	1.0	1.0	1.0
120-2y	1.0	1.0	1.5	2	1.0	1.5	1.0	1.5	1.0	1.0	1.0	2.0
67y	1.0	1.5	2.5	2.5	1.0	1.0	1.5	2.5	1.0	1.0	1.0	1.0
61y	1.0	1.0	1.5	2.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
116y	1.0	1.0	1.5	2.5	1.0	1.5	1.5	1.5	1.0	1.5	1.5	2.0
126y	1.0	1.5	1.5	2	1.0	1.5	2.0	2.0	1.0	1.0	1.0	1.0
34y	1.0	1.0	1.0	2.5	1.0	1.5	1.5	2.0	1.0	1.5	1.5	3.0
20-1y	1.0	1.5	2	2.5	1.0	1.5	1.5	2.5	1.0	1.5	1.5	2.5
36-1y	1.0	1.0	1.0	1.5	1.0	1.5	1.5	1.5	1.0	1.0	1.0	1.0
113y	1.0	1.0	1.0	1.5	1.0	1.5	2.0	2.0	1.0	1.0	1.0	1.5
114-3y	1.0	1.0	1.0	1.5	1.0	1.5	2.0	2.0	1.0	1.0	1.0	1.0
64y	1.0	1.0	1.0	1.5	1.0	1.0	1.5	1.5	1.5	2.0	2.0	2.0
41y	1.0	1.0	1.0	1.0	1.0	1.5	1.5	2.5	1.0	1.0	1.0	1.0
112By	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	2.5	2.5	3.0
30-3y	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	2.5	2.5	3.0
108-2y	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.0	1.5	1.5	1.0
6By	1.0	1.0	2.0	2.0	1.0	1.0	1.0	1.0	1.0	2.0	2.0	2.5
20-2y	1.0	1.0	1.0	1.5	1.0	1.0	1.5	1.5	1.0	1.5	1.5	1.5
40y	1.0	1.0	1.0	1.0	1.0	1.0	2.0	2.0	1.0	2.0	2.0	3.0
4y	1.0	1.0	1.0	1.5	1.0	1.0	2.5	2.5	1.0	2.0	2.0	2.5
control	1	1.0	2.0	2.5	2.5	1.5	2.5	3.0	1.5	4.0	4.0	4.5
Mean	1.02	1.09	1.35	1.72	1.06	1.23	1.58	1.81	1.09	1.60	2.07	2.67
LSD (5%)	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	1.19	1.26	1.39

Disease rating was based on 1-5 scale for detached leaf tests where, 1=1-25%, 2=26-50%, 3=50-70%, 4= 70-90% and 5=90-100% (ICARDA, 1986)

CS20DK and 47-2y, 67y, 61y, 126y 113y, 64y, 108-2y on local cultivar. Reduction in disease severity on the local check cultivar was more prominent and significant. Disease severity was constantly delayed on CS20DK and EH0013-18 cultivars during the observation period.

The disease severity scoring result of the cultivars showed low levels of disease pressure compared to the control. Disease initiation was not inhibited on the local susceptible check, but development after 48 hr was reduced significantly compared to control. Isolates, isolate 108-2y, 20-2y, 47-2y and 36-1y proved most effective in reducing disease development on all the three cultivars.

DISCUSSION

This study has revealed that the biological control agents for chocolate spot of faba bean and *Bacilli* are natural residents of faba bean leaves in Ethiopia. Occurrence of *Bacillus* species on 110 leaf samples collected from 12 districts clearly indicates their wide prevalence. Differences in colony characters and growth rates showed the variability in *Bacillus* populations in north-west Ethiopia. Thirty eight distinct isolates were found to occur on faba bean leaves. Maximum variability was observed in Kutaber district, from where 9 distinct isolates were recovered. *Bacillus* species have been reported to occur on wheat and grapes also and some isolates have been found to be effective for control of foliar diseases (Kildea *et al.*, 2008).

Laboratory studies showed the presence of significant inhibitive potential in isolates of *Bacillus* against *B. fabae*. Isolate 43y produced largest inhibition zone under the dual culture (8 mm), while isolates 111-1y and 116y caused largest lysis of pathogen mycelium, when inoculated on young colony of pathogen. Isolates 111-1y and 116y were confirmed from CABI Global Plant Clinic as *Bacillus* and not pathogenic to plants and animals. The other 15 isolates also produced significant inhibition zones and 9 isolates had a significant degree of pathogen mycelium lysis.

Several studies have established that *Bacillus* isolates possess potential for controlling this serious pathogen. Gnanamanickam and Mew (1992) reported high inhibition zone by *Bacillus* strains 4-03 and 33 against *Pyricularia oryzae*, a causative of blast disease of rice. Sharma and Sharma (2008) also found that *B. subtilus* produced antifungal compounds, which caused morphological alterations in the vegetative cells and spores, and disruption and lysis of cell walls of *Alternaria* species causing leaf spot of mustard. *Bacillus megaterium* and *Pseudomonas fluorescens* have been found to reduce biomass of *Mycoshaerella graminicola* by 43% or more in dual culture (Kildea *et al.*, 2008).

This study has revealed that the *Bacillus* spp. were well adapted for nutrient and better survivors against the pathogen in controlling the chocolate spot pathogen. Campbell (1994) suggested that screening must select for organisms adapted to the environment in which they are to operate. According to Sherga (1997), *Bacillus* isolates can be used as a biocontrol agent against *Botrytis fabae* and *Botrytis cinarea*. Antagonists can control the diseases by competing for essential nutrients, antibiosis and direct parasitism. Their secondary metabolites can cause disruption of spores and mycelium lysis. *B. pumilus* has been reported to cause lyses of germ tubes of uredospores of three cereal rusts (Morgan, 1963).

Evaluation of 20 isolates of *Bacillus*, which had shown significant antagonistic potential *in vitro*, further proved their effectiveness against the disease on detached leaves. Most of them reduced disease development, when inoculated 24 hr after pathogen inoculation. After 5 days of inoculation, the disease was almost contained by 41y, 12By, 3-3y and 108-2y on susceptible cultivar EH0013-18; 61y and 6By on tolerant cultivar CS20DK; and 47-2y, 67y, 61y, 126y, 36-1y, 114-3y 41y and 108-2y on susceptible local check. It also reflected that the isolates had different potential of controlling the disease on varieties having different levels of resistance against the disease. However, isolates 108-2y, 47-2y, 36-1y, and 20-2y were able to control disease development equally across all the three cultivars equally.

Isolate 108-2y produced a moderate inhibition zone and caused significant lysis of mycelium *in vitro*. It had moderate growth rate and reduced the growth of pathogen also moderately;

however, it proved to be the most effective on detached leaves.

Isolate 47-2y showed high inhibition zone and lysis of pathogen and proved very effective on detached leaves, but had moderate growth rate and pathogen growth reducing capacity. Isolate 20-2y produced high inhibition zones and pathogen mycelium lysis, had high growth rate and reduced growth of *B. fabae* significantly. Such observations have earlier also been reported by some workers (Sherga, 1997).

Bacillus species have a number of characteristics useful for biocontrol of plant diseases. They form endospores, which can withstand ecological stresses like high temperature and moisture stress for long periods. They are resistant to ultraviolet and gamma radiations, desiccation, lysozymes, starvation and chemical disinfectants (Thompson, 2007). They can survive well in soil as well as on leaves. *B. subtilus* has been mainly studied for biocontrol potential and occasionally *B. megaterium, B. cerus, B. pumilus* and *B. polymixa* have been tried (Silo-Suh *et al.*, 1994).

Bacillus pumilus has been found to cause lyses of germ tubes of uredospores of three cereal rusts (Morgan, 1963). Bacillus has been exploited less for commercial utilisation than other bacteria for disease control, but now products are being developed. In U.K. product Serenade, (based on *B. subtilus*) is being registered for use against *B. cinerea* on strawberry (DHDC, 2008). Our studies have shown that all these four isolates have high potential of controlling chocolate spot of faba bean and can be further evaluated for commercial utilization, either alone or as a component of integrated disease management.

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