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# POTENTIAL ANTAGONISTIC FUNGAL SPECIES FROM ETHIOPIA FOR BIOLOGICAL CONTROL OF CHOCOLATE SPOT DISEASE OF FABA BEAN

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## ABSTRACT

Chocolate spot disease (*Botrytis fabae* Sard) is one of most yield limiting constraints of faba bean (*Vicia faba*). There is promise in using biological control agents to control chocolate spot diseases, nevertheless, this strategy has not been fully exploited. The objective of this study was to assess the prevalence of different antagonistic fungi on phyloplane of faba bean in Ethiopia and to evaluate their antagonistic potential against the pathogen. A total of 110 isolates of *Trichoderma* species were obtained from faba bean leaves from 12 districts, which were grouped into 18 distinct groups differing in colony and other characters. Similarly, 26 distinct isolates belonging to species of *Penicillium, Aspergillus, Fusarium* and *Phioalophora* were identified from leaves of faba bean. *In vitro* and *in vivo* studies revealed strong antagonistic potential of many isolates. Thirteen isolates of *Trichoderma* produced 4 mm or more inhibition zone and reduced growth of pathogen colony, when grown in dual culture with it. Antagonistic isolates caused lysis of pathogen mycelium more than 6 mm on agar plates. The antagonists significantly reduced pathogen growth in a range of 24.5 to 0.8 mm. The efficacy of the *Trichoderma* isolates ranged from 47.6 to 98% and that of the other fungal isolates ranged from 13.1 to 34.5%. On detached leaves, isolates 6-1T, 18-3T and 87T of *T. ovalisporum* and 52-BT, 108-1T and 108-4T of *T. longibrachiatum* were found to reduce development of chocolate spot on four genotypes of faba bean. The outcome indicates that biocontrol agents, particularly of species *Trichoderma* are prevalent on faba bean leaves and can be further explored and developed into effective mycofungicides for management of chocolate spot disease of faba bean.

*Key Words*: Aspergillus *Botrytis fabae, Trichoderma, Vicia fabae*

## RÉSUMÉ

La maladie de tâche du chocolat (*Botrytis fabae* Sard) est une des contraintes limitatives du rendement du haricot faba (*Vicia faba*). L'utilisation des agents biologiques de contrôle serait promettant, par ailleurs, cette stratégie n'a jamais été amplement exploitée. L'objectif de cette étude était d'évaluer la prévalence de différents champignons anatagonistiques sur la phylloplane du haricot faba en Ethiopie et d'évaluer leur potentiel antagonistique contre le pathogène. Un total de 110 isolats d'espèces de *Trichoderma* était obtenu des feuilles du haricot faba dans 12 districts, et groupées en 18 groupes différents en colonie et autres caractères. Similairement, 26 différents isolats appartenant aux espèces de *Penicillium, Aspergillus, Fusarium* et *Phioalophora* étaient identifiées des feuilles de haricot faba. Des études i*n vitro* et *in vivo* ont révélé un fort potentiel anatagonistique de beaucoup d'isolats.Treize isolats de *Trichoderma* ont produit 4 mm ou plus de zones d'inhiition et ont réduit la croissance de colonies pathogéniques lorsque cultivés ensemble. Sur plateaux agar, des isolats anatagonistiques ont causé plus de 6 mm de *lysis* des mycelium pathogéniques. Les anatagonistes ont significativement réduit de 24.5 à 0.8 mm la croissance des pathogènes. L'efficacité des isoltas de *Trichoderma* variait entre 47.6 et 98% et celle d'autres isolats fongiques variait entre 13.1 et 34.5%. Sur des feuilles détachées, les isolats 6-1T, 18-3T et 87T de *T. ovalisporum* et 52-BT,

108-1T et 108-4T de *T. longibrachiatum* étaient trouvées susceptibles de réduire le développement de la tâche du chocolat sur quatre génotypes du haricot faba. Le résultat indique que des agents biocontôles, particulièrement des espèces *Trichoderma* sont prévalants sur des feuilles de haricot faba et peuvent être examinés et développés en mycofungicides efficaces pour la gestion de la maladie de tâche du chocolat du haricot faba.

*Mots Clés*: Aspergillus *Botrytis fabae*, *Trichoderma*, *Vicia fabae*

## INTRODUCTION

Faba bean (*Vicia fabae* L.) is one of the most important food legumes due to its high nutritive value both in terms of energy and protein contents (24-30 %) and is an excellent nitrogen fixer (Sahile *et al*., 2008a). Ethiopia is the third largest producers of faba bean in the world, next to china and Egypt (Torres *et al*., 2006). Faba bean is grown on 370,000 hectares in Ethiopia with an annual production of about 450,000 tonnes (ICARDA 2006). Despite its wide cultivation, the average yield of faba bean is quite low in Ethiopia, because of many biotic and abiotic constraints (Sahile *et al*., 2008a).

Chocolate spot is the most important disease of faba bean worldwide and is capable of devastating unprotected crops up to 67% (Bouhassan *et al*., 2004). It appears as reddish brown spots on leaves and under favorable conditions on stems, flowers and pods. Subsequently, these spots grow larger and can even merge into black mass. The disease results in heavy premature defoliation and under warm moist conditions crop lodging may occur. Plant growth and most physiological activities are adversely affected leading to drastic reduction in yield (Khaled *et al*., 1995). This disease is caused by *Botrytis fabae* Sard. and *B. cinerea Pers.Fr*. (Harrison 1988). In Ethiopia chocolate spot is caused by *B. fabae* and occurs almost regularly in most faba bean growing areas (Dangachew, 1967). The disease can reduce faba bean yields up to 67% (Bouhassan *et al*., 2004).

Different management options have been developed to reduce the yield losses in faba bean due to chocolate spot worldwide. These include the use of chemical fungicides, resistant/ tolerant varieties, use of certain cultural practices such as crop residue management and altering planting date (Dereje, 1999; Bretag and Raynes, 2004; Hawthorne, 2004). There is weak genetic resistance in cultivars of faba beans to chocolate spot (Lawes *et al*., 1983) and the most common control strategy is fungicide sprays. However, the negative effects of fungicide use are already becoming apparent. For instance, development of resistance in *B. cinerea* and in *B. fabae* against fungicides has been reported (Parry, 1990). Management options recommended in Ethiopia for this disease are application chlorothalonil or mancozeb and late planting (Dereje, 1993; Sahile *et al*., 2008b ) but have not been adopted by the farmers at large. Only one resistant variety, CS20DK was released in Ethiopia 20 years ago, but it did not become popular because of lower yield, and subsequently loss of resistance (Gemechu *et al*., 2006).

Biological control is another option, which has not been fully exploited. It is economical, self-perpetuating and usually free from residual effects and can be an important component of integrated disease management. Faba bean phyloplane harbours many microorganisms of different groups, besides the chocolate spot pathogen because of its high proteins content. Some of them might be antagonistic to *B. fabae*. Sherga (1997) found that out of 270 isolates of *Bacillus* tested, 14% had strong antagonistic effect against chocolate spot pathogen in *vitro*. However, fungal antagonists have not been explored for biological management of this serious disease of this important food legume.

The objective of this study was to identify the potential fungal antagonists to *B. fabae* from Ethiopia, which can be developed into commercial mycofungicides for the integrated management of chocolate spot disease of faba bean.

## MATERIALS AND METHODS

**Collection of leaf samples.** Samples of healthy looking leaves were collected from faba bean plants having disease on other leaves, for exploring the resident antagonistic mycoflora

of healthy faba bean leaves. Such samples were collected from farmers' fields of twelve districts of Amhara Regional State in north western Ethiopia. This region is located between longitude 36 - 40° W to E, and latitudes 11 - 13° 45' south-west to north. Similarly leaves aggressively affected by chocolate spot were collected from faba bean plants from a farmer's field in Kutaber district for isolation of virulent pathogen. All the leaf samples were kept in folds of newspapers in a plant press for 48 hr and, thereafter, kept securely in labelled paper bags till isolation of the microorganisms.

**Isolation of faba bean resident mycoflora and pathogen.** The collected leaves of faba bean were surface sterilised in 1% sodium hypochlorite and subsequently washed three times in sterilised distilled water. From decontaminated leaves 5-mm$^2$ pieces were cut with sterile scalpel and placed on potato dextrose agar (PDA) in 9 cm diameter culture dishes. These were incubated for one week at 21° C±1 and the fungi emerging from leaf tissues were transferred to PDA and purified. Fungal isolates in pure cultures were coded and transferred to screw-capped culture bottles containing faba bean extract dextrose agar and potato dextrose agar (FBEA/PDA) and stored at 4 °C. The *B. fabae* was isolated from diseased leaves on PDA, purified, identified and stored at 4 °C as stock culture of pathogen.

**Cultural characteristics of isolates.** The isolates of all fungi were grown on PDA in culture dishes at 21° C ±1 for 96 hr. Characteristics like colony colour and diameter, morphological and sporulation of all the isolates were compared and recorded (Dhingra and Sinclair, 1986). Generic level identification of isolates was done and broadly classified into *Trichoderma* isolates and other fungal isolates. Different isolates within both groups showing similar colony characteristics were again grouped together and one representative isolate of each subgroup was taken for antagonistic potential studies. Cultural characteristics like growth rate, colony colour and diameter and medium reverse colour of all representative isolates of *Trichoderma* and other fungi were studied at 21°C on PDA. Isolates of *Trichoderma* that showed promising antagonistic activity were identified from CAB International Global Plant Clinic, London, UK.

**Antagonistic activity in fungal isolates against *B. fabae*.** The antagonistic activity of different fungal isolates from apparently healthy leaves was tested against *B. fabae* firstly *in vitro* and then *in vivo.*

The fungi isolated from the leaves were tested for antibiosis activity to *B. fabae* on PDA in 9 cm Petri dishes. Three petri-dishes were inoculated with 2-mm mycelial disc from the edges of an actively growing colony of *B. fabae* on one side and with a similar sized disc of fungal isolate on the other side and incubated at 21° C ±1. After 72 hr of growth the inhibition zones at the junction of colonies of fungal isolates and *B. fabae* were measured using verner calipper.

In order to test for *lysis of B. fabae colony* 4 ml of *B. fabae* mycelial disc was placed on PDA (15 ml) in 9 cm petri-dish and incubated at 21 °C ± 1. After three days of mycelial growth 2-mm agar disc of the potential fungal isolates of *Trichoderma*, and other fungal isolates from actively growing colonies were placed on the colony of *B. fabae* and incubated at 21°C ± 1. Lysis of *B. fabae* colony was examined periodically under stereomicroscope (50x) and the width of lysed mycelia around the colony of the lytic fungal isolate was measured. The experiments were replicated three times in completely randomized design. Culture plates with *B. fabae* alone were used as the control.

Six *Trichoderma* isolates showing fast growth, significant inhibition zone and lytic activity were identified at CAB International Global Plant Clinic and used in the *in vivo* test of the antagonistic potential. Detached-leaves of four faba bean varieties namely CS20DK (tolerant), EH91011-6-2 (moderately resistant) and EH0013-18 (susceptible check) and one local check were used *in vivo* testing using the aggressive *B. fabae* culture according to the Paul *et al*. (1995) procedure. Leaves without antagonist evidence were used as the control.

Leaves of faba bean of the same age group were sterilised with 70% ethanol and placed in

sterile 15 mm petri-dishes having sterilised filter paper moistened with sterile water. A spore suspension of *B. fabae* (2.5 x $10^5$ spores $ml^{-1}$) was prepared according to Mohammed *et al*. (1994). One drop (1 ml) of the spore suspension was placed near the midrib. The covered petri-dishes serving as moist chamber were incubated at 20 °C. After 24 hours, a drop of potential antagonist having 2.0 x $10^5$ cfu $ml^{-1}$ was added to the pathogen at the midrib and incubated at 20 °C again. The experiment was arranged in a complete randomised design.Disease reaction was recorded at 48, 72, 96 and120 hr of inoculation of antagonist using a 1-5 scale for detached leaf test (ICARDA, 1986).

**Statistical analysis.** Analysis of variance was carried out using SPSS V.12. Measurement data from *in vivo* test using detached leaves severity scales were subjected to SAS (Ver. 8). Mean comparisons were made using the Least Significant Difference test at $P<0.05$ test.

## RESULTS

**Isolation of resident mycoflora.** From 1044 leaves collected from 12 districts, *Trichoderma* was isolated from 110 samples, showing 10.5% frequency of occurrence (Table 1). *Trichoderma* species was found to occur on faba bean leaves in all the districts; however, distribution of these isolates was found to vary among the districts. Highest number (13) of isolates was obtained from Kutaber district, followed by Hulet Eju Ensae. The elevation of the sampled areas varied between 1900 and 3319 meters above sea level.

Fungi other than *Trichoderma* species also appeared in isolations made from the 1044 leaves. Predominant fungi species observed on faba bean leaves were *Penicillium, Aspergillus* and *Fusarium*. Species of *Penicillium* were highly frequent and occurred on leaf samples from several areas. *Aspergillus niger* was isolated from 7 districts namely Debark, Desei Zuria, Kutaber, Farta, Wogera, Yilmana Densa and

TABLE 1. Natural occurrence of *Trichoderma* species on faba bean leaves in the districts of northwestern Ethiopia

| Distrıct | Altitude range (m.a.s.l) | No. of *Trichoderma* isolates obtained | Potential antagonistic *Trichoderma* isolates | Potential antagonistic other fungal isolates | % potential *Trichoderma* isolates within each districts |
|---|---|---|---|---|---|
| Yilmana Densa | 1980-2405 | 9 | 6-1T | 10-p2(P. sp), 2(A. n) | 5.6 |
| Hulet Eju Ensae | 2275-2670 | 11 | 14bT, 18-2T, 18-3T, | 14(P. sp), 18-1(A. n) | 16.7 |
| Gonder Zuria | 1969-2463 | 8 | - | 29(P. sp), 25(A. fl), 24(F. sp) | 0 |
| Wogera | 2650-2943 | 9 | - | 30-1(A. n) | 0 |
| Debark | 2740-3053 | 7 | - | 49(A. n) | 0 |
| Farta | 1975-3000 | 11 | 51-bT, 52B-2T | 56(A. n) | 11.2 |
| Lay Gaint | 2794-3184 | 10 | 63T, | 62(P. sp), 68(A. fl) | 5.6 |
| Meket | 2779-3319 | 9 | - | - | 0 |
| Gubalafto Woldia | 1900-3033 | 8 | 87 | 81(P. sp) | 5.6 |
| Ambasel Tehuledrae | 1908-2196 | 8 | 108-1T, 108-3T, 108-4T, | 108-2(P. sp), 104(F. sp) | 16.7 |
| Kutaber | 2144-3250 | 13 | 114-3T, 117-2, 118T, 120-2T, 122-1T | 119-2(P. sp), 117B(P. sp), 119-B(P. sp), 122-2(A. n), 112(A. n), 126(A. fl), 130-2(F. sp), 130-1(F. Sp), 120-2y1(Ph. sp) | 33.3 |
| Desei Zuria | 2055-3138 | 7 | 140-2 | 134-2(P. sp), 140-3(A. n) | 5.6 |

[1]. -, No isolate obtained. [2]. A. fl = *Aspergillus flavus* ; A. n = *Aspergillus niger* ; F. sp = *Fusarium* sp. ; P. sp = *Penicillium* sp. ; Ph. sp = *Phailophora* sp.

Hulet Eju Ensae while; *A. flavus* occurred in Kutaber, Lay Gaynt and Gonder Zuria districts. Similarly, *Fusarium* sp. occurred in Ambasel Tehuledrae, Gonder Zuria and Kutaber districts. *Phailophora* sp. was found to occur on faba bean leaves in Kutaber districts only (Table 1).

**Cultural characteristics of isolates of *Trichoderma* species and other fungi.** Cultivation of fungal isolates on PDA under similar conditions showed that some isolates of the same genus resembled each other in colony characteristics, mycelium and sporulation characters while differed from other such groups of the same genus (Table 2). Eighteen distinct groups of *Trichoderma* were found to occur within its total 110 isolates from faba bean leaves. Further studies on PDA with one representative isolate of each group showed that colony diameters of 18 *Trichoderma* isolates varied from 39.4 to 61.55 mm after 96 hr of growth. Isolates 18-2T, 18-3T, 51b-T, 52-2T, 87T, 108-1T, 108-3T, 108-4T, 114-3T, 117-2T, 118T, 120-2T and 140-2T had fairly high growth rates. Isolates 51-bT, 108-3T, 108-4T, 117-3T and 118T grew as suppressed colonies; while all others had raised aerial growth. Isolates 6-1T, 14-bT, 63T, 87T and 118T had white to green colour; while 51bT and 52-2T had grey to yellow colour (Table 2). All other isolates of *Trichoderma* were green in colour. There were distinct differences in media reverse colour of isolates, which varied from white, yellow, yellowish green to green. Species of *Penicillium* exhibited relatively slow growth rates with colony diameter ranging from 27.1- 31.2 mm in 96 hr of growth on PDA in comparison to *A. niger* and *A. flavus* with colony diameter of 32.2 - 48.4 and 33.2 – 37.2 mm, respectively. *Phailophora* sp. attained 41.3 mm colony diameter in the same period (Table 3).

**Antibiosis activity in fungal isolates.** Dual culture studies on PDA for evaluation of antibiosis activity of *Trichoderma* species and other fungi revealed that all inhibited growth of *B. fabae* by degrees and exhibited inhibition zone at the junction with the pathogen. Out of 18 *Trichoderma* isolates tested, 13 isolates viz., 6-1T, 14bT, 18-3T, 52-2T, 87T, 108-1T, 108-3T, 108-4T, 117-2T, 118T, 120-2T, 122-1T and 140-2T

TABLE 2. Colony and growth characteristics of different *Trichoderma* species isolates from north western Ethiopia

| District | Isolate code | Altitude (m.a.s.l) | Colony diameter (mm) | | | Colony | | Media reverse colour |
|---|---|---|---|---|---|---|---|---|
| | | | 48 hr | 72 hr | 96 hr | Type | Colour | |
| Yilmana Densa | 6-1T | 2702 | 15.33 | 23.00 | 39.45 | A | W/g | W |
| Hulet Ejue Ensae | 14bT | 2424 | 17.78 | 28.33 | 44.60 | A | W/g | G |
| Hulet Ejue Ensae | 18-2T | 2544 | 21.11 | 31.67 | 47.30 | A | G | G |
| Hulet Ejue Ensae | 18-3T | 2544 | 20.00 | 30.00 | 46.20 | A | G | Y |
| Farta | 51-bT | 2727 | 25.56 | 38.33 | 58.40 | S | G/y | W |
| Farta | 52-2T | 2702 | 23.78 | 35.67 | 51.67 | A | G/y | W |
| Lay Gaynt | 63T | 2145 | 18.89 | 28.33 | 44.33 | A | W/g | W |
| Gubalafto Woldia | 87T | 2810 | 22.22 | 33.33 | 49.45 | A | W/g | W |
| Ambasel Tehlederae | 108-1T | 2003 | 16.00 | 24.00 | 50.00 | A | G | G |
| Ambasel Tehlederae | 108-3T | 2003 | 23.33 | 35.00 | 51.23 | S | G | W |
| Ambasel Tehlederae | 108-4T | 2003 | 30.00 | 45.00 | 61.55 | S | G/y | Y |
| Kutaber | 114-3T | 2264 | 25.56 | 38.33 | 58.30 | A | G | G |
| Kutaber | 117-2T | 2407 | 23.33 | 35.00 | 51.33 | A | G | G/y |
| Kutaber | 117-3T | 2407 | 18.89 | 28.33 | 44.00 | S | G | G |
| Kutaber | 118T | 2216 | 25.56 | 38.33 | 58.00 | S | W/g | W |
| Kutaber | 120-2T | 2505 | 25.56 | 38.33 | 58.33 | A | G | G |
| Kutaber | 122-1T | 2567 | 16.67 | 25.00 | 41.45 | A | G | W |
| Dessei Zuria | 140-2T | 2784 | 25.56 | 38.33 | 58.25 | A | G | W |

A = aerial; S = suppressed; W/g = white to green; G/y = Green to yellow; G = green; W = white; Y = yellow; G/y = gray to yellow

TABLE 3. Colony and cultural characteristics of different fungal isolate

| District | Fungi | Isolate code | Altitude (m. a. s. L.) | Colony growth diameter (mm) | | Colony | Character at 96 hr |
|---|---|---|---|---|---|---|---|
| | | | | 72 hr | 96 hr | Colour | Media reverse colour |
| Kutaber | *Penicillium* sp. | 119-2 | 2430 | 15.33 | 31. 21 | B/g | Y |
| Lay Gaynt | *Penicillium* sp. | 62 | 2100 | 12.00 | 28.17 | B/g | B/g |
| Hulet Eju Ensae | *Penicillium* sp. | 14 | 2424 | 14.00 | 30.19 | B/g | G |
| AmbaselTehulederae | *Penicillium* sp. | 108-2 | 2003 | 11.33 | 27.16 | B/g | W |
| Gonder Zuria | *Penicillium* sp. | 29 | 2075 | 14.67 | 30.20 | B/g | W |
| Desei Zuria | *Penicillium* sp. | 134-2 | 2679 | 12.67 | 28.17 | Blue | Y |
| Gubalafto Woldia | *Penicillium* sp. | 81 | 3020 | 11.00 | 27.15 | Blue | B |
| Kutaber | *Penicillium* sp. | 117B | 2407 | 15.33 | 31.21 | B/g | W |
| Yilmana Densa | *Penicillium* sp. | 10p-2 | 2360 | 14.00 | 30.19 | B/g | W |
| Kutaber | *Penicillium* sp. | 119-B | 2430 | 15.00 | 31.21 | B/g | W |
| Debark | *Aspergillus niger* | 49 | 3122 | 25.67 | 41.36 | Br | W |
| Dessei Zuria | *Aspergillus niger* | 140-3 | 2784 | 23.00 | 39.32 | Br | W |
| Kutaber | *Aspergillus niger* | 122-2 | 2567 | 24.00 | 40.33 | Br | W |
| Farta | *Aspergillus niger* | 56 | 2851 | 23.00 | 39.32 | Br | W |
| Kutaber | *Aspergillus niger* | 112 | 2131 | 25.00 | 41.35 | Br | W |
| Wogera | *Aspergillus niger* | 30-1 | 2241 | 16.08 | 32.22 | Br | W |
| Yilmana Densa | *Aspergillus niger* | 2 | 2443 | 24.00 | 40.33 | Br | W |
| Hulet EjuEnsae | *Aspergillus niger* | 18-1 | 2544 | 32.67 | 48.45 | Br | W |
| Lay Gaynt | *Aspergillus flavus* | 68 | 2731 | 21.00 | 37.29 | Y | W |
| Kutaber | *Aspergillus flavus* | 126 | 2336 | 17.67 | 33.25 | Y | W |
| Gonder Zuria | *Aspergillus flavus* | 25 | 2268 | 21.00 | 37.29 | Y | W |
| Gonder Zuria | *Fusarium* sp. | 24 | 2647 | 23.00 | 39.32 | W/r | W/r |
| Ambasel Tehulderae | *Fusarium* sp. | 104 | 2679 | 22.00 | 38.31 | W/r | W/r |
| Kutaber | *Fusarium* sp. | 130-2 | 2838 | 24.33 | 4034 | W/r | W |
| Kutaber | *Fusarium* sp. | 130-1 | 2838 | 24.00 | 40.33 | P | P |
| Kutaber | *Phailophora* sp. | 120-2y1 | 2505 | 25.56 | 41.36 | O | W |

B/g = blue to green; G = green; B = blue; Br = brown; Y = yellow; W = white; W/r = white to red; P = pink; O = orange

produced 4 mm or higher inhibition zone (Table 4). Isolates of *Trichoderma* species reduced the growth of *B. fabae* colony by varying degrees. All *Penicillium* isolates produced 4-5 mm inhibition zones. *Aspergillus niger* isolates produced 5-6 mm inhibition zones in comparison to 4-5 mm by *A. flavus* and *Fusarium* species. Single isolate of *Phailophora* sp. produced 6 mm inhibition zone against *B. fabae* (Table 5).

**Lytic potential in fungal isolates.** All the *Trichoderma* isolates when placed on mycelium of *B. fabae* caused lysis to varying extent. Isolates 6-1T, 52-2T, 87T, 108-1T, 108-3T and 120-2T caused 8-10 mm of lysis around them. *Trichoderma* isolates 6-1, 18-2, 18-3, 51-b, 52-2, 63, 87, 108-1, 108-3, 108-4, 114-3, 117-2, 117-3, 118, 120-2,122-1 and 140-2 overgrew upon the pathogen mycelium (Table 4).

Species of *Penicillium* placed on *B. fabae* also caused lysis of its mycelium, which ranged from 6.7 – 11.2 mm. Isolates 119-2, 62, 14, 29, 134-2, 81, 10-p-2 and 119-B caused higher lysis ranging from 9-11.2 mm. Species of *Aspergillus* produced lysis ranging from 6-12.5 mm and isolates 49, 140-3, 122-2, 56, 112, 30-1, 18-1 and 2 of *A. niger* and 68 of *A. flavus* showed higher lytic potential. Except for isolate 130-2, *Fusarium* species produced lesser lysis of mycelium of pathogen. *Phailophora* sp. proved effective in lysing the pathogen by 9.5 mm (Table 5).

TABLE 4. Effect of *Trichoderma* species on growth *Botrytis faba*

| Isolate code | Inhibition zone(mm) | Lyses (mm) | Efficacy (%) | *Trichoderma* species colony growth at 72 hr (in mm) | Mean growth of *B. fabae* at 72 hr (in mm) |
|---|---|---|---|---|---|
| 6-1T | 5 | 10.25 | 50.2 | 23.00 b | 22.8ab |
| 14bT | 4 | 7.00 | 61.8 | 28.33 ab | 17.47b |
| 18-2T | 2 | 7.00 | 69.2 | 31.67 ab | 14.13b |
| 18-3T | 4 | 7.00 | 65.5 | 30.00 ab | 15.8b |
| 51-bT | 3 | 7.00 | 83.7 | 38.33 ab | 7.47c |
| 52-2T | 5 | 10.25 | 77.9 | 35.67 ab | 10.13bc |
| 63T | 2 | 7.00 | 61.9 | 28.33 ab | 17.47b |
| 87T | 4 | 10.25 | 72.8 | 33.33 ab | 12.47bc |
| 108-1T | 4 | 10.25 | 47.6 | 24.00 b | 21.8ab |
| 108-3T | 5 | 8.00 | 76.4 | 35.00 ab | 10.8bc |
| 108-4T | 4 | 7.00 | 98.3 | 45.00 a | 0.8d |
| 114-3T | 3 | 7.00 | 83.7 | 38.33 ab | 24.47a |
| 117-2T | 4 | 7.00 | 76.4 | 35.00 ab | 10.8bc |
| 117-3T | 2 | 7.00 | 61.9 | 28.33 ab | 17.47c |
| 118T | 5 | 6.25 | 83.7 | 38.33 ab | 7.47c |
| 120-2T | 4 | 10.25 | 83.7 | 38.33 ab | 7.47c |
| 122-1T | 4 | 7.00 | 54.6 | 25.00 b | 20.8ab |
| 140-2T | 4 | 7.00 | 83.7 | 38.33 ab | 7.47c |
| Mean | 3.78 | 7.14 | 72.1 | 33.02 | 12.78 |
| Control | | | 0 | | 45.80 |
| LSD (5%) | NS | NS | | 18.59 | 6.59 |

NS = non significant. Over growth has bean occurred during 96 hr growth

**Effect of Trichoderma species on chocolate spot *in vivo*.** Effect of 3 isolates 6-1T, 18-3T and 87T belonging to *T. ovalisporum* and 3 isolates 52-BT, 108-1T and 108-4T of *T. longibrachiatum* on development of chocolate spot on four genotypes of faba bean was studied *in vivo* using detached leaf technique (Table 6). All isolates were found to reduce chocolate spot severity, when inoculated with the pathogen. However, their effect varied with isolate and genotype. Isolates 108-1T, 108-4T and 52-BT, of *T. longibrachiatum* were the most effective in reducing the mean disease severity on all the four genotypes and provided 43-47% mean disease control. Out of three isolates of *T. ovalisporum*, 6-1T proved better than other isolates of this species and reduced the disease by 40%, but was less effective than those of *T. longibrachiatum*. Isolates 18-3T and 87T of *T. ovalisporum* were very effective on susceptible genotypes EH91011-6-2, EH0013-18 and local check, but failed to reduce severity on moderately resistant genotype CS20DK. Highly significant disease control (75%) was provided by *T. ovalisporum* (isolates 108-1T and 108-4T) on genotype EH0013-18. Highest disease pressure developed on local check and all the 6 isolates could reduce disease severity. In general, all the isolates effectively reduced the disease on the two susceptible genotypes, but were less effective on moderately resistant genotypes EH91011-6-2 and CS20DK.

## DISCUSSION

*Trichoderma* species predominantly occurred on faba bean leaves in Ethiopia. Species of *Trichoderma* were encountered from 10.5% leaf samples from 12 districts, indicating their natural adaptability to faba bean leaves. However, a variation in isolates of this genus was also found to be widespread. Within the total 110 isolates of *Trichoderma* species obtained from leaves, 18 distinct isolates showing clear differences in

TABLE 5. *In vitro* effect of fungal isolates on the growth of *Botrytis fabae*

| Fungi | Isolate code | Inhibition zone (mm) | Lyses (mm) | Efficacy (%) | 72 hr growth diameter (mm) of fungal | Botrytis mean radial growth (mm) at 72 hr |
|---|---|---|---|---|---|---|
| *Penicillium* sp. | 119-2 | 4b | 11.00 b | 30.5 | 15.33 b | 33.47cd |
| *Penicillium* sp. | 62 | 5ab | 10.75 ab | 33.8 | 12.00 b | 26.2dc |
| *Penicillium* sp. | 14 | 5ab | 9.25 ab | 31.8 | 14.00 b | 30.57cd |
| *Penicillium* sp. | 108-2 | 4b | 6.75 b | 34.5 | 11.33 b | 24.74d |
| *Penicillium* sp. | 29 | 5ab | 11.00 ab | 31.1 | 14.67 b | 32.03cd |
| *Penicillium* sp. | 134-2 | 4b | 10.75 b | 33.1 | 12.67 b | 27.66dc |
| *Penicillium* sp. | 81 | 5ab | 11.25 ab | 34.8 | 11.00 b | 24.02d |
| *Penicillium* sp. | 117B | 4b | 6.75 b | 30.5 | 15.33 b | 33.47cd |
| *Penicillium* sp. | 10p-2 | 5ab | 10.50 ab | 31.8 | 14.00 b | 30.57cd |
| *Penicillium* sp. | 119-B | 4b | 11.25 b | 30.8 | 15.00 b | 32.75cd |
| *Aspergillus niger* | 49 | 4b | 11.00 b | 20.1 | 25.67 ab | 56.05a |
| *Aspergillus niger* | 140-3 | 4b | 12.00 b | 22.8 | 23.00 ab | 50.22bc |
| *Aspergillus niger* | 122-2 | 5ab | 10.50 ab | 21.8 | 24.00 ab | 52.41bc |
| *Aspergillus niger* | 56 | 5ab | 11.00 ab | 22.8 | 23.00 ab | 50.22bc |
| *Aspergillus niger* | 112 | 6a | 10.00 a | 20.8 | 25.00 ab | 54.59b |
| *Aspergillus niger* | 30-1 | 6a | 11.00 a | 29.7 | 16.08 b | 35.11cd |
| *Aspergillus niger* | 2 | 5ab | 11.25 ab | 21.8 | 24.00 ab | 52.41bc |
| *Aspergillus niger* | 18-1 | 6a | 12.50 a | 13.1 | 32.67 a | 71.33a |
| *Aspergillus flavus* | 68 | 4b | 6.00 b | 24.8 | 21.00 ab | 45.85cb |
| *Aspergillus flavus* | 126 | 4b | 7.00 b | 28.13 | 17.67 ab | 38.58c |
| *Aspergillus flavus* | 25 | 5ab | 9.25 ab | 24.8 | 21.00 ab | 45.85cb |
| *Fusarium sp.* | 24 | 5ab | 5.00 ab | 22.8 | 23.00 ab | 50.22bc |
| *Fusarium sp.* | 104 | 5ab | 4.50 ab | 23.8 | 22.00 ab | 48.03bc |
| *Fusarium sp.* | 130-2 | 4b | 11.25 b | 21.47 | 24.33 ab | 53.67bc |
| *Fusarium sp* | 130-1 | 4b | 6.75 b | 21.8 | 24.00 ab | 52.41bc |
| *Phailophora sp.* | 120-2y1 | 6a | 9.50 ab | 20.24 | 25.56 ab | 55.81b |
| Control | | | | | | 45.80 |
| LSD (5%) | | 2.39 | 1.939 | | 16.35 | 16.35 |

colonies and morphology were established. Besides, *Trichoderma*, 26 distinct isolates of *Penicillium*, *Aspergillus*, *Fusarium* and *Phailophora* were also found to be prevalent on faba bean leaves. A total of 11 isolates belonging to *A. niger* and *A. flavus*, 10 isolates of *Penicillium*, 4 isolates of *Fusarium* and one isolate of *Phialophora* were obtained from faba bean leaves. There was no co-relation of species of fungi with altitude as all of them occurred at all altitudes from where samples were collected. This clearly indicated their wide adaptability to different environments. Goldfarb *et al.* (1989) reported the varying nature of the growth rate of *Trichoderma* with species and temprature. In their study, Goldfarb and his co-workers found the growth rate of *Trichoderma* spp. to vary from 12.7-23.4 mm $day^{-1}$ depending on the species at 20 $^0$C of temprature. In another experiment, Saber *et al.*, (2009), found daily growth rate of different fungal antagonist in the range of 15-35 mm/day. In their experiment conducted in Egypt, the author reported that all of the fungal antagonists tested showed reasonably higher growth rate than the pathogen *B. fabae*.

The dual culturing of pathogen with 18 isolates of *Trichoderma* and 26 of other fungi revealed clearly potential of control in some of the isolates. Thirteen isolates of *Trichoderma* produced 4 mm or higher inhibition zone on agar medium. These might be producing antibiotics or extracellular enzymes, which inhibited growth of the pathogen. Similar strong antagonistic behaviour of some isolates was observed in

TABLE 6. Effect of *Trichoderma* species on development of chocolate spot on genotype of faba bean

| *Trichoderma* species | Isolate no. | Faba bean genotype | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EH91011-6-2 | | | 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 | 48 hr | 72 hr | 96 hr | 120 hr |
| *T. ovalisporum* | 6-1T | 1 | 1 | 1 | 1.5b | 1.5cb | 2bac | 2bac | 2.5bac | 1.5 | 1.5ba | 1.5 | 1.5 | 1c | 1cb | 2.5b | 2.5b |
| *T. ovalisporum* | 18-3T | 1 | 1 | 1 | 1b | 2b | 2.5ba | 2.5ba | 3ba | 2 | 2ba | 2 | 2 | 1c | 1c | 2b | 2b |
| *T. ovalisporum* | 87T | 1 | 1 | 1 | 1.5b | 1c | 1.5bc | 1.5bc | 2bc | 2 | 2ba | 2.5 | 2.5 | 1c | 1c | 2.5b | 2.5b |
| *T. longibranchiatum* | 52BT | 1 | 2 | 2 | 2ba | 1c | 1c | 1c | 1c | 1.5 | 1.5ba | 1.5 | 1.5 | 1.5cb | 1.5cb | 3b | 3b |
| *T. longibranchiatum* | 108-1T | 1 | 1 | 1 | 1.5b | 1c | 1c | 1c | 1c | 1.5 | 1.5ba | 1.5 | 1.5 | 2b | 2b | 3b | 3b |
| *T. longibranchiatum* | 1108-4T | 1 | 1 | 1 | 1.5b | 1c | 1.5bc | 1.5bc | 1.5bc | 1 | 1b | 1.5 | 1.5 | 2b | 2b | 2.5b | 2.5b |
| | Control | 2 | 2 | 2 | 3a | 3a | 3a | 3a | 4a | 2 | 2ba | 2 | 2 | 3a | 4a | 5a | 5a |
| | LSD (5%) | NS | NS | NS | 1.5 | 1.00 | 1.19 | 1.19 | 1.73 | NS | 1.84 | NS | NS | 0.65 | 0.99 | 1.94 | 1.94 |

[1]Disease rating was based on 1-5 scale for detached leaf test where, 1 = 1-25%, 2 = 26-50%, 3 = 51-70%, 4 = 71-90% and 5 = 91-100% area affected (ICARDA, 1986) [2] NS = non significant

lysing the pathogen mycelium in agar plates. Some isolates proved effective in antibiosis as well as in lysis, while some others were better in antibiosis and some better in lysing. This reflects the differences in the spectrum and degree of their antibiotic and enzyme production. The genus *Trichoderma* comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms. Elad and Stewart (2004) have also reported that *Trichoderma, Gliocladium* and *Ulocladium* have greatest potential for *Botrytis* diseases and commercial success has been achieved in glasshouse and post-harvest environments for disease control. In the activity of biological control, micro-organisms action is not limited to direct influence on the target diseases, in addition to their direct effect they also enhance the resistance of the plants. A report by Benítez *et al.* (2004) indicates that *Trichoderma* strains are known to promote plant growth and plant defensive mechanisms and antibiosis against, the pathogen or direct mechanisms such as mycoparasitism. *T. harzianum* and *T. viridi* have been reported as biocontrol agents for chocolate spot of grape, apple and strawberry caused by *B. cinerea* (Sutton *et al.*, 1997; Hjeljord *et al.*, 2001).

The study showed that there were promising antagonistic species of fungi prevalent on faba bean leaves, which can be exploited for the control of chocolate spot. Although, different genera of fungi were found to have antagonistic ability against the pathogen *in vitro*, from the point of view of wider antagonistic spectrum, *Trichoderma* species were considered more feasible for further exploration. Therefore, isolates 6-1T, 18-3T and 87T of *T. ovalisporum* and 52-BT, 108-1T and 108-4T of *T. longibrachiatum* were further tested *in vivo* by detached leaf technique. All of them were found to reduce the development of chocolate spot on two susceptible and two moderately resistant genotypes of faba bean, though degree of reduction varied and also depended on genotype of faba bean. Isolates provided a higher extent of control in susceptible genotypes. These antagonistic isolates were fast growing and

reduced the colony growth of *B. fabae*, when grown in dual culture.

This study has revealed isolates of *T. ovalisporum* and *T. longibrachiatum* as effective antagonists of *B. fabae* for the first time. *Trichoderma ovalisporum* is an endophytic type of fungus and was first identified as a new and novel biocontrol agent from Amazon basin of South America for frosty pod rot (*Moniliopthora rori*) and wittches' broom (*Crinipellis* spp.) of cocoa (Holmes *et al*., 2004; Holmes *et al*., 2006).

*Trichoderma* strains are known to control pathogens either indirectly by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly by mechanisms such as mycoparasitism (Benítez *et al*., 2004). *Trichoderma* species such as *T. harzianum, T. viridi* and *T. polysporum* are well known antagonists. Ten commercial products of these three species were developed for controlling diseases on different crops (Frevel *et al.,* 1998). *T. harzianum* and *T. viridi* were reported as biocontrol agent for chocolate spot of grape, apple and strawberry caused by *B. cinerea* (Sutton *et al*., 1997; Hjeljord *et al*., 2001).

Other fungi prevalent on faba bean leaves also exhibited antagonistic activities against *B. fabae in vitro*. Isolates 62, 29, 10-p2 of *Penicillium*; 140-3, 122-2, 56, 18-1 and 112 of *A. niger*, 25 of *A. flavus* and 24 of *Fusarium* and 120-2yl of *Phailophora* caused wide inhibition zone and lysis of mycelium. The antagonists evaluated in this study showed significant differences in reducing pathogen growth and their effects ranged from 24.47 to 0.8 mm (Table 5). Earlier also *Penicillium brevicompactum* and *Cladosporium cladosporioides* isolated from faba bean leaves were found to have significant antagonistic activity against *B. fabae in vitro* and *in vivo* (Jackson *et al*., 1997). Commercial products like Biofox C and Fusaclean having non-pathogenic strains of *Fusarium oxysporum* have been developed for controlling soil borne diseases (Frevel *et al.,* 1998). De Cal *et al.* (2008) reported biological control of powdery mildew on strawberry leaves by *Penicillium oxalicum* applications, it was achieved on different cultivars and lines in growth chambers and in open-field nurseries. Species of *Penicillium*, *Aspargillus* and *Fusarium* have been reported by Leibinger *et al*. (1997) as an antagonists against *Botrytis cinerea*. Mass production technology by solid state fermentation for conidi of *Penicillium frequentans*, a biocontrol agent of the fungal pathogen *Monilinia laxa* has been developed by using specially designed plastic bags (VALMIC ® ) containing peat and vermiculite (De Cal *et al*., 2002 ).

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