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Field Performance of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on Faba Bean (*Vicia faba* L.) Growth Promotion and Management of Chocolate Spot (*Botrytis fabae* Sard.)

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Authors' contributions

This study was conducted in collaboration among the authors of this study. Author ZF designed the study, worked the field experiment, collected all the data, performed the statistical analysis and prepared the first draft of the manuscript. Author FA read, re-wrote and approved the manuscript. Author TA read, edited and approved the manuscript.

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ABSTRACT

Aims: This study was done to evaluate the effects of *T. harzianum* AAUT14 and *B.subtilis* AAUB95 on chocolate spot (*B. fabae*) and growth promotion of faba bean.
Study Design: A completely randomized block design was utilized.
Place and Duration of Study: The study was conducted at Kulumsa Agricultural Research Center, 8º2'N and 39º10'E, Kulumsa, June-November, 2018.
Methodology: Two trails (Trial-1 Ashebeka and Trial-2 Hachalu) were employed. We included T1-Control (B.f only); T2-*T. harzianum* AAUT14+ B.f; T3-*B. subtilis* AAUB95+B.f; T4-*T. harzianum* AAUT14+*B. subtilis* AAUB95+B.f; T5- MORE 720 WP+B.f; T6- ORZEB+B.f as treatments of the study. The disease development was assessed together with yield and related parameters.

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Results: In trail 1, a reduction that varied from 31-61% for disease incidence and 13-33% of severity over T1 upon 70 days after sowing. Upon 90 days, the disease incidence and severity was reduced to 20-50% and 36-51%, respectively. *Trichoderma harzianum* AAUT14+*B.subtilis* AAUB95 (T4) reduced the disease incidence and severity showing no significance difference (*P*=.05) with the chemical fungicide, MORE 720 WP (T5) upon 70 and 90 days of sowing. In trial 2, the microbial inoculants reduced the disease incidence and severity to 28-63% and 17-30% upon 70 days. Likewise, the disease incidence and severity was reduced to 23-51% and 37-54% upon 90 days. In addition, the AUDPC ranged from 1586.1-2250.0%DSU in trial 1 and 1382.0-2454.5%DSU in trial 2. Moreover, leaf area of 68.95cm^2 was displayed by T4 in trial 1 and 54.14cm^2 in trial 2. In addition, T 4 indicated, 62% and 49% increment of hundred seed dry weight and grain yield estimate in trial 1, and 56% and 55%, increase in trial 2 compared to the uninoculated control. The percentage of healthy pods was 90% and 88.87% in trial 1 and 2, respectively, in the treatment that received T4 and followed by T2 that showed 70.40 and 78.86% in trial 1 and 2, respectively. T4 resulted 4391.45kg/ha and 4378.12kg/ha, that followed by T2 with 3764.58kg/ha and 3654.17kg/ha of yield estimate in trial 1 and 2, respectively. 27-42% and 26-41% of harvest index was exhibited in trial 1 and trial 2, respectively. Furthermore, the seed nitrogen content increased from 33-70% in trial 1 and 29-62% in trial 2. The seed nitrogen content showed 33-70% and 29-62% increment in trial 1 and 2, respectively. Even though the fungicides (T5 and T6), protected the faba bean plants from chocolate spot, there was <10% of seed nitrogen and crudeprotein content increment over the untreated control plants in both varieties.
Conclusion: The mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 or in some cases *T. harzianum* AAUT14 performed best on controlling chocolate spot, growth promotion and yield increment of faba bean.

Keywords: Bio-agents; co-inoculation; crude protein; fungicides; harvest index; leaf area; yield.

1. INTRODUCTION

Foliar fungal diseases such as chocolate spot (*Botrytis fabae*), alternaria leaf spot (*Alternariaalternata*), rust (*Uromycesfabae*), and downy mildew (*Peronospora viciae*) are the causative agents of yield losses and its components in faba bean [1]. Of these diseases, chocolate spot is the major problem of faba bean plants in Tunisia, Algeria, Morocco, Libya, Ethiopia and other countries such as Spain, Norway, Germany, Scotland, Russia, Japan, China, Canada and Australia [2]. The disease is capable of devastating the unprotected faba bean, result in a harmful effect on plant growth, physiological activities and yield of the crop [3] and sometimes complete crop failures [4].

The use of fungicides against chocolate spot has offered good results, but the rise of chemical fungicides and its negative impact on the environment necessitates the use of ecofriendly approaches to manage this disease. Biological control is one of the most important method being utilized for controlling many fungal diseases of plants and the search for potent microbe is also increasing as potential biological control agents [5] and [6]. *Trichoderma* and *Bacillus* spp. are the best microbial member that act as biological control agents of chocolate spot under *in vitro* and in vivo conditions [7]. Thus, the application of *Bacillus* and *Trichoderma* species as bio-control agents have received much attention for sustainable agricultural activity in many countries. In addition, these microbes have the ability to produce phytohormones, antifungals, solubilize and provide the insoluble nutrients to the host plants [8].

In faba bean, the mixture of *B. subtilis* and *T. viride* increased the yield by 33% compared to the uninoculated control once in the presence of faba bean rust infection as biotic stress [9]. Similarly, the application of different *T. harzianum* strains under field conditions resulted an increase of yield from 8-30% beside acting as the biocontrol agents of chocolate spot [10]. Seed treatment of bio-agents combined with foliar treatments were more effective for controlling leaf spot of faba bean than foliar spray only [11]. Moreover, a mixture of *Rhizobium leguminosarum* and *T. viride* tag4 as seed treatment and foliar spray increased the yield by 23% compared to the uninoculated control plants beside controlling chocolate spot [3]. Additionally, the treatment improved the physiological activities (photosynthetic pigments, total phenol and polyphenol oxidase) and growth of the plant. However, the combined use of antagonistics (*Bacillus* and *Trichoderma* spp.) as biological

control agents of chocolate spot is not fully evaluated yet under field conditions.

In our previous study, two strains (*T. harzianum* AAUT14 and *B. subtilis* AAUB95) were found the best candidates for their antagonistic property against *B. fabae* and plant growth-promoting traits under in vitro study. In addition, the combination of the strains showed better bio-control of chocolate spot and improved faba bean growth under in vivo (greenhouse) conditions. Therefore, this study was conducted to further evaluate the efficacy of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 when separately and co-inoculated on chocolate spot management under naturally infested field condition and the performance of faba bean.

2. MATERIALS AND METHODS

2.1 Study Site

This study was conducted at Kulumsa Agricultural Research Center (KARC) in Ethiopia, at the time of faba bean growing season, June-November, 2018. KARC is located at 8º2'N and 39º10'E coordinate, having 2200m above sea level and annual rain fall of 840mm in Tiyo district of Arsi zone, Kulumsa. The site is mandated to highland pulse crops mainly faba bean and cereals such as wheat, and barley research. This field is naturally infected with *B. fabae*, the causal agent of faba bean`s chocolate spot.

2.2 Sources of Bio-agents, Faba Bean Varieties and Fungicides

Trichoderma harzianum AAUT14 and *B. subtilis* AAUB95 (antagonistic agents) were obtained from our previous studies. The rhizobial strain, *R. leguminosarumbv*. viciae (FB-1035) was taken from Holleta Agricultural Research Center and the two faba beans (Ashebeka and Hachalu) varieties were obtained from KARC. The fungicides, viz, MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80WP (Mancozeb) were bought from local market.

2.3 Inoculum Preparation

The inoculum of *T. harzianum* AAUT14 was prepared according to [12]. A 5mm mycelial disc was inoculated on fresh potato dextrose agar (PDA) and incubated at 25±2°C for five days, after incubation 10mL sterile water was added to the plates, the suspension was filtered through two layers sterilized gauze, the spore suspension was collected into flasks and adjusted to the concentration of 2.5×10^5 spore mL^{-1} using haemocytometer [13]. *Bacillus subtilis* AAUB95 was cultured in 100mL flasks containing 40mL nutrient broth. The flasks were incubated on an orbital shaker (ZJZD-III, Shanghai, China) at 130rpm for 48hr. and $1x10^9$CFU mL^{-1} of cells were utilized for the experiment [14].

2.4 Seed Coating, Foliar Spray of the Bio-agents and Fungicides

Faba bean seeds, were washed with tap water, surface sterilized by 1.5% sodium hypochlorite for 1min and rinsed in distilled-sterilized water thoroughly. Seeds were treated with *T. harzianum* AAUT14 having 2.5×10^5 spore mL^{-1}[13], *B. subtilis* AAUB95 with $1x10^9$CFU mL^{-1}[14] and *R. leguminosarumbv*, viciae (FB-1035) containing $1x10^9$ CFU mL^{-1}[15] as seed coating using 10% carboxyl methyl cellulose each at a rate of 10mL/kg of seed, air dried and sown directly [16]. In addition, foliar spraying of the developed plants with the same bio-agents (*T. harzianum* AAUT14 and *B. subtilis* AAUB95) were done on the 35th and 55th days after sowing with 3mL/plant [3] and the fungicides, MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80WP (Mancozeb) were applied following the instruction given on the packs.

2.5 Experimental Layout and Treatments

The plots were prepared with $3.2m^2$ (4 x 0.8m) area, 60cm distance between plots, 40cm distance between rows, 4m length and 1.5m apart between blocks. In the experiment, two trails having six (6) treatments were done separately for two faba bean varieties (Trial-1 Ashebeka and Trial-2 Hachalu variety). The experimental layout had three blocks and six rows within block. The treatments were applied with three replications using a completely randomized block design (CRBD) in a zig zag pattern. Triple super phosphate (TSP) was applied according to [17]. All the agronomic practices were done manually with the involvement of man power. Accordingly, the following treatments were allocated in the experiment: - T1-Control (*Botrytis fabae* only); T2-*Trichoderma harzianum* AAUT14+*Botrytis fabae*; T3-*Bacillus subtilis* AAUB95+*Botrytis fabae*; T4-*Trichoderma harzianum* AAUT14+*Bacillus subtilis* AAUB95+ *Botrytis fabae*; T5- MORE 720 WP+ *Botrytis fabae*; T6-ORZEB 80WP + *Botrytis fabae*.

2.6 Disease Assessment and Data Collection

After 70 days of planting, the treatments were assessed for disease development in terms of disease incidence and severity. Disease incidence was expressed as a percentage of infected leaves out of the total leaves per treatment following the early stage of symptoms development. Disease severity was expressed as percent of affected leaf based on symptoms appeared according to [18] using a rating scale of 0-5 (0= no symptoms,1= up to 5%, 2=6-10%, 3=11-25%, 4=26-50% and 5=51-100% of leaf area affected. The scale (1-5) was rated to infected leaves on the basis affected areas` disease strength through visual observations. An area under disease progress curve (AUDPC) was calculated according to [19].

%DS= Sum of all diseases/Total number of ratings x maximum disease grade x100

%DI = Total No. of diseased leaves/Total No. of leaves per treatment x 100

$$AUDPC=\sum_{i=1}^{n} (Y_i+Y_i+1/2)(t_i+1-t_i)$$

Where n= total number of observations, Y_i= injury intensity (usually incidence in crop health data) at the i^{th} observation, and t= time at the i^{th} observation. Since the unit for Y in the sample data is % and the unit for t is development stage, the unit of the AUDPC is, % development stage unit [19].

Disease reduction percentage (%R) per treatment was calculated according to [16] using the following formula: -

$$\%R=[1-(DT/DC)] *100$$

Where,

DT-disease incidence/severity percentage in the treatment and DC- disease incidence/severity percentage in the control.

2.7 Experimental Data Collection

The collected parameters were leaf length, leaf width, leaf area, pod number and the percentage of healthy pods. The percentage of healthy pod was calculated by considering diseased pods (showing symptom of chocolate spot). The succeeding model proposed by [20] was used to calculate the leaf area using randomly taken leaf samples from each treatment.

$$LA = 0.919 + 0.682*L*W$$

Where,

LA- leaf area (cm^2), L- maximum leaf length (cm) and W- maximum leaf width (cm).

On harvest, the treatments were checked for the number of seeds per pod, hundred seed dry weight (g), the yield obtained from each plot was converted to kg/ha for analysis and the percentage of harvest index (HI) was calculated according to [21].

$$\%\text{HI} = \frac{\text{Grain yield of treatment (g)x100}}{\text{Shoot dry weight of treatment (g)}}$$

2.8 Seed Nitrogen Content Analysis and Crude Protein Estimation

Seed nitrogen content was analyzed according to Kjeldahl method. Two hundred milligram (200mg) of dried seed sample was taken in a 100mL Kjeldahl flask, 5mg of salt mixture (potassium sulphate, cupric sulphate and selenium powder mixed in the ratio of 50:10:1) was added with 3mL of concentrated sulphuric acid, followed by digestion, after digestion, 10mL of distilled water was added. The distillate was collected in a conical flask having 10mL of 4% boric acid and 3 drops of mixed indicator (0.3g bromocresol green and 0.2g methyl red in 400 ml of 90% ethanol) and titrated against 0.05N HCl. The crude protein was calculated by multiplying the total nitrogen content (%) of seed by Jone`s conversion factor (i.e. 6.25) according to [22].

$$\text{Nitrogen (\%)} = \frac{\text{Sample titre} - \text{blank titre} \times \text{Hot HClx14x 100}}{\text{Sample weight x 1000}}$$

2.9 Statistical Analysis

The data were analyzed by General Linear Model (GLM) univariate analysis of variance (ANOVA) using randomized completely block design (RCBD). Mean values were separated by Duncan multiple range test (DMRT) and Tukey`s HSD analysis at α= 0.05 by SPSS version 24 and all the values were considered significant at $P<.05$.

3. RESULTS AND DISCUSSION

In the present study, the inoculation of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 was

done under field conditions to evaluate their potential against chocolate spot under naturally infested conditions. The treatment exhibited different levels of disease incidence and severity along with area under disease progress curve as a function of days after sowing. In Ashebeka variety (Trail 1), a reduction that varied from 31-61%, 13-33%, was recorded for disease incidence and severity over control upon 70 days after sowing, respectively. In the same trend, the disease incidence and severity was reduced in the range of 20-50% and 36-51%, respectively upon 90 days after sowing (Table 1). This shows the existence of variation among and between the treatment applied to each experimental unit in controlling the pathogen (*B. fabae*) and its respective disease (chocolate spot). The dual application of bio-agents, *T. harzianum* AAUT14+*B.subtilis* AAUB95 (T4) showed reduction of disease incidence and severity showing no significance difference (*P=.05*) with the chemical fungicides, MORE 720 WP(T5) upon 70 and 90 days of sowing. This may indicate the efficiency of the strains to be used as the effects of fungicide (T5) in biological control of chocolate spot disease depending on their antagonistic property. On the other side, *T. harzianum* AAUT14 (T2) performed better than *B. subtilis* AAUB95 (T3) that showed less efficacy in this study. This finding is in agreement with [23] who reported that the effectiveness of *Trichoderma* spp. than *B. megaterium* in controlling chocolate spot under field study.

Best efficacy of *T. harzianum* than *B. subtilis*was reportedin controlling faba bean fungal pathogen, *F. solani* in common beans and chickpea [17,24]. This could be attributed to the mechanisms through which *T. harzianum* AAUT14 or *B. subtilis* AAUB95 antagonize *B. fabae*, the same trend of performance was also displayed by the strain under the greenhouse conditions in our previous study. The performance difference might be due to the diversity of mechanisms exerted by *T. harzianum* AAUT14 and that could have an additive effect in plant protection. However, the combination of T2 and T3 indicated better performance whichis comparable with the chemical fungicides (T5 and T6). The combination of *T. harzianum* T5- and *B. subtilis* Bs1 strains provided the best reduction of*Fusarium* infection along itsdisease severity and incidence in comparison to the individual treatments in chick pea [17]. In this regard, [25] demonstrated that the efficiency of biological control agents in mixtures was related to complementary modes of action exhibitedby the combined microorganisms.

The bio-agents and chemical treatments also reduced the disease incidence and severity on the Hachalu variety, in trial 2 as presented below (Table 2). The bio-agent reduced disease incidence and severity in the range of 28- 55%% and 17-37% upon 70 days of sowing, respectively. Likewise, the disease incidence and severity was reduced to the range of 23-46% and 37-48% in 90 days of sowing by the bio-agents, respectively. This shows as the application of bio-agents reduced the disease incidence and severity compared to the control (T1) in both trials. Similarly, the disease severity reduction of chocolate spot in faba beans treated by bio-control agents, *Trichoderma* and *Bacillu*s spp. compared with untreated faba beans [25]. The T4 inoculated plants showed the highest reduction ofdisease incidence especially at the latter days (90 days) of sowing(48%) compared to T5 (54%) and T6(44%) treated plants. This indicates as the performance of T4 was in between the chemical fungicides, T5 and T6 used in the presentstudy, in which the same trend of performance was also observed in trial 1.

In addition, AUDPC that ranged from 1586.1-2250.0%DSU and 1382.0-2454.5%DSU was shown by the different treatments in trial 1 (Table 1) and trial 2 (Table 2), respectively. The maximum AUDPC observed was 2250.0%DSU and 2454.5%DSU in T1 of trial 1 and 2, respectively. Fungicides unsprayed faba bean, showed an AUDPC of 1817%DSU,1476%DSU, 1467%DSU and 1716%DSU of AUDPC on Sinana local, Shallo, Mosissa and Walki varieties of faba bean, respectively [26]. Their finding is different from this study which might be caused by the difference in the varieties used and or due to the inoculum size of the pathogenic fungi found in the study fields. Nevertheless, in this study, T4 demonstrated 1660.1%DSU and 1382.0%DSU of AUDPC in trial 1 and 2, respectively. Thus, this indicates as the chocolate spot symptom development was significantly influenced by the treatments.

The applicationofboth antagonists eitherindividually andor in combination not only controlled the chocolate spot, but also promoted plant growth. In this aspect, the treatments indicated variability in leaf area that ranged from 20-68.95cm^2 in trial 1 and 20.11- 54.14cm^2 in trial 2 as shown below (Table 3). All the treatments

showed significant difference with the untreated control in both trials, in which the antagonist inoculated faba bean plants (either separately and or in combination) showed best leaf area than the fungicides (T5 and T6) treated once. An area of 68.95cm^2 was displayed by T4 and followed by T2 that showed 49.05cm^2 in trial 1. Correspondingly, in trail 2, 54.14cm^2 and 50.02cm^2 of leaf area was shown by T4 and T2, respectively. This may reveal the extra role of bio-agents supplied to the host plants such as the synthesis of phytohormones and or nutrient solubilization beside acting as the biological control agents of chocolate spot. In plants, an increase of leaf area enhances the rate of photosynthesis and the largest leaf area is an indicator of best growth and productivity in crops [20].

Moreover, 120 days after sowing, all the treatments showed significantly different number of pods and chocolate uninfected (healthy pods) compared to T1 in both trials as shown below (Table 4). This may indicate the potential of the treatments to control chocolate spot under naturally infested field conditions. The percentage of healthy pods was 90% and 88.87% in trial 1 and 2, respectively, in the treatment that received T4 and followed by T2 that showed 70.40 and 78.86% in trial 1 and 2, respectively. T2 showed the same trend of protecting faba bean plants from chocolate spotshowing no significant variation with T6 in both varieties (trial 1 and 2). Additionally, the combination of T2 with T3, T4 resulted a good performance of protecting faba bean pods from chocolate spot following MORE 720 WP. This might be related to the antagonistic property such as hydrogen cyanide (HCN), and lytic enzymes (protease and lipase) displayed by T3 and the mycoparasitic potential of T2 as determined in our previous *in vitro* study. This may also indicate the ability of the treatment to act as bio-fungicides in addition to growth-promoting agents of faba beans.

On the other side, pod number and seeds per pod was also varied from treatments to treatments. The number of pods and seeds per pod was maximum in both trials that received T4 compared to the other treatments and followed by T2. Furthermore, T2 showed a comparable result with T5 in these aspects. However, T3 indicated less performance in both trial which might be dealt with potency of the strain when inoculated lonely. The combined application of *T. harzianum* with *R. leguminosarum* increased the growth parameters viz. the number of pods and seeds per pod in the presence of biotic stress induced by *Fusarium* sp. in field grown faba bean [27,28].

Following harvest, both the fungicides and bio-agents' treatment showed significant difference in both trials with respect to yield and yield relatedparameters of the control, T1 (Table 5). Faba bean plants treated with the fungicide, Diathane M45 (Mancozeb) and biocides, *B. subtilis* and *T. harzianum* gave the highest seed yield per plot, hundred seed weight (g) and seed yield ton per fed. as compared with the uninoculated control [29]. In the present study, the treatments, T4 resulted significant variation (*P=.05*) to all the treatments with regard to the considered parameters. In T4 received experimental unit, there was 62%, and 49% increment of hundred seed dry weight and grain yield estimate in hectare over T1, respectively in trial 1. The same treatment showed 56% and 55%, increase of hundred seed dry weight and grain yield estimate in hectare, respectively in trial 2. However, in both cases, the fungicides (T5 and T6) treated plants showed a comparable result to that of T2 in enhancing the same parameters. Faba bean plants sprayed with the mixture of *B. subtilis* and *T. viride* increased the yield by 33% compared to the unsprayed once in the presence of faba bean rust infection as biotic stress [9]. T2 showed similar trend of better faba bean yield increment following the combined inoculation (T4). The treatment illustrated 21% and 22% yield increase in trial 1 and 2, respectively. The different *T. harzianum* strains under field conditions showed an increase of faba bean yield from 8-30% beside acting as the bio-control agents of chocolate spot [10]. Likewise, *T. viride* tag4 mixed with *R. leguminosarum* increased the yield of faba beans by 23% compared to the uninoculated control plants [3]. In this study, T4 also showed no significance difference (*P=.05*) in yield improvement compared to one of the fungicides utilized in this study (T5). This may indicate the major role of *T. harzianum* AAUT14 when combined with *B. subtilis* AAUB95 and *R. leguminosarum* bv, viciae (FB-1035) to improve the faba bean production through overcoming the influence induced by chocolate spot. According to [27] the combined application of *T. harzianum* with *R. leguminosarum* increased the plant growth parameters viz. mean seed dry weight and seed yield of faba bean in the presence of biotic stress induced by *Fusarium* sp. in field study.

Table 1. The effect of separate or together application of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 on faba bean (Ashebeka) chocolate spot(*B. fabae*) disease incidence and severity under field conditions

Treatments	Days after sowing								AUDPC (%DSU)
	70				90				
	%DI	%R	%DS	%R	%DI	%R	%DS	%R	
T1-Control(B.fonly)	23.80[a]	-	40.00[a]	-	58.00[a]	-	75.00[a]	-	2250.0[a]
T2-*T. harzianum* AAUT14+B.f	14.00[c]	40	32.13[c]	20	36.60[c]	37	44.11[c]	41	1886.77[c]
T3-*B. subtilis* AAUB95+B.f	16.43[b]	31	35.00[b]	13	46.37[b]	20	48.31[b]	36	2058.1[b]
T4-*T. harzianum* AAUT14+*B.subtilis* AAUB95+B.f	10.80[e]	55	28.00[e]	30	29.26[e]	44	38.00[e]	47	1660.1[d]
T5-MORE 720 WP+B.f	9.24[e]	61	27.00[e]	33	28.89[e]	50	37.11[e]	51	1586.1[e]
T6-ORZEB 80WP+B.f	11.59[cd]	51	28.31[d]	29	32.26[c]	44	42.00[cd]	44	1693.9[d]
CV	0.35	-	0.15	-	0.27	-	0.27	-	0.17

B.f- Botrytis fabae, DI-Disease incidence, DS-Disease severity, %R- Percentage of reduction over T1, AUDPC- Area under disease progress curve,DSU- Development stage unit and CV-Coefficient of variation. Mean values of three replications within same columns labeled with same letter (s) of superscript are not significantly different (P=.05) according to DMRT and Tukey HSD analysis of Two-Way ANOVA

Table 2. The effect of separate or together application of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 on faba bean (Hachalu) chocolate spot(*B. fabae*) disease incidence and severity under field conditions

Treatments	Days after sowing								AUDPC (%DSU)
	70				90				
	%DI	%R	%DS	%R	%DI	%R	%DS	%R	
T1-Control (B.fonly)	22.17[a]	-	40.10[a]	-	55.56[a]	-	65.00[a]	-	2454.5[a]
T2-*T. harzianum* AAUT14+B.f	14.00[b]	37	31.00[c]	23	36.46[c]	34	39.25[bc]	40	1787.67[b]
T3-*B. subtilis* AAUB95+B.f	16.00[a]	28	33.10[b]	17	42.58[b]	23	41.14[b]	37	1900.9[b]
T4-*T. harzianum* AAUT14+*B.subtilis* AAUB95+B.f	9.04[d]	55	25.30[d]	37	30.17[de]	46	34.00[cd]	48	1478.5[cd]
T5-MORE 720 WP+B.f	8.15[d]	63	24.00[d]	40	27.20[d]	51	30.20[cd]	54	1382.0[d]
T6- ORZEB 80WP+B.f	11.00[c]	50	25.40[d]	37	33.16[cd]	40	36.50[bc]	44	1508.0[c]
CV	0.38	-	0.19	-	0.27	-	0.29	-	0.21

B.f- Botrytis fabae, DI-Disease incidence, DS-Disease severity, %R- Percentage of reduction over T1, AUDPC- Area under disease progress curve, DSU- Development stage unit and CV-Coefficient of variation. Mean values of three replications within same columns labeled with same letter (s) of superscript are not significantly different (P=.05) according to DMRT and Tukey HSD analysis of Two-Way ANOVA

Table 3. The effect of single and dual inoculation of antagonistic *T. harzianum* AAUT14 and *B. subtilis* AAUB95 on faba bean leaf length (cm), width (cm) and area (cm^2)

Treatments	T1-Ashebeka			T2-Hachalu		
	LL (cm)	LW (cm)	LA (cm^2)	LL (cm)	LW (cm)	LA (cm^2)
T1-Control (B.fonly)	8.30^{d}	3.37^{c}	20.00^{d}	8.71^{d}	3.23^{c}	20.11^{d}
T2-*T. harzianum* AAUT14+B.f	13.50^{ab}	5.80^{ab}	54.32^{b}	13.50^{ab}	5.55^{a}	50.02^{ab}
T3-*B. subtilis* AAUB95+B.f	13.00^{b}	5.77^{ab}	49.05^{bc}	13.00^{b}	5.46^{a}	48.33^{bc}
T4-*T. harzianum* AAUT14+*B. subtilis* AAUB95+B.f	14.25^{a}	7.00^{a}	68.95^{a}	14.59^{a}	5.37^{a}	54.14^{a}
T5- MORE 720 WP+B.f	11.00^{c}	4.51^{bc}	34.75^{c}	12.81^{b}	4.72^{ab}	33.30^{c}
T6- ORZEB 80WP+B.f	11.66^{c}	4.60^{bc}	37.23^{c}	11.00^{c}	4.33^{b}	36.45^{c}
CV	0.17	0.26	0.38	0.17	0.19	0.31

B.f- Botrytis fabae, T1-Trial 1, T2- trial 2,LL-Leaf length, LW-leaf width, LA-Leaf area. Mean values of three replicas in the same columns labeled with same letter (s) of superscript are not different (P=.05) according to DMRT and Tukey HSD using Post Hoc analysis of Two-Way ANOVA

Table 4. The effect of single and dual application of antagonistic *T. harzianum* AAUT14 and *B. subtilis* AAUB95 on faba bean pod number and symptoms of chocolate spot (*B. fabae*) development on pods

Treatments	T1-Ashebeka			T2-Hachalu		
	NP	NSPP	%HP	NP	NSPP	%HP
T1-Control (B.f only)	9.53^{d}	1.80^{c}	36.86^{e}	10.07^{d}	1.83^{c}	40.07^{e}
T2-*T. harzianum* AAUT14+B.f	13.40^{b}	2.57^{a}	70.40^{c}	14.17^{b}	3.10^{a}	78.86^{c}
T3-*B. subtilis* AAUB95+B.f	12.00^{c}	2.27^{b}	60.52^{d}	12.77^{c}	2.00^{b}	66.54^{d}
T4-*T. harzianum* AAUT14+*B. subtilis* AAUB95+B.f	15.00^{a}	2.60^{a}	90.00^{b}	16.73^{a}	3.30^{a}	88.87^{b}
T5- MORE 720 WP+B.f	12.40^{b}	2.22^{ab}	94.25^{a}	13.67^{c}	2.27^{ab}	92.39^{a}
T6- ORZEB 80WP+B.f	11.00^{c}	2.00^{bc}	80.45^{c}	12.70^{c}	2.12^{bc}	75.31^{c}
CV	0.15	0.46	0.28	0.17	0.45	0.25

B.f- Botrytis fabae, T1-Trial 1, T2-Trial-2, NP-Number of pods, NSPP-Number of seed per pod, %HP- Percentage of healthy pods. Mean values of three replicas within same columns labeled with same letter (s) of superscript are not different (P=.05) according to DMRT and Tukey HSD Post Hoc analysis of Two-Way ANOVA

Furthermore, harvest index was higher than T1 in both trials that received one of the treatments. This may show the effect of either single and or dual application of the antagonistic strains on faba bean yield improvement. 27-42% and 26-41% of harvest index improvement was exhibited in trial 1 and trial 2, respectively by one of the employed treatments. T4 resulted 4391.45kg/ha and 4378.12kg/ha, that followed by T2 with 3764.58kg/ha and 3654.17kg/ha of yield estimate in trial 1 and 2, respectively. This could probably indicate the possibility of yield enhancement through inoculating plant beneficial microorganisms in faba bean crops. The highest harvest index was indicated by the treatment that showed the maximum yield estimates. The highest grain yield, of 4886.8kg/ha was found in Shallo and 4362.2kg/ha in Mosissa variety of faba beans with the highest value of harvest index, 45 and 43%, respectively [30]. After harvest, seed nitrogen and crude protein content indicated variation from the uninoculated control faba bean plants. The seed nitrogen content increased from 33-70% and 29-62% in trial 1 and 2, respectively. Even though the fungicides (T5 and T6) protected the faba bean plants from chocolate spot, there was negligible increment of seed nitrogen content in both trials over T1 (<10%). Similarly, the crude protein of seed increased from 33-70% in trial 1 and 29-66% in trial 2 through the application of the bio-agents. Similar to the seed nitrogen content, the fungicides showed negligible increment of seed crude protein in both trials over T1 (<10%).

Therefore, this study demonstrated the effects of biotic stress, fungal pathogens on faba bean nutrient accumulation after harvest and the synergy plant beneficial bacterial and fungal groups to overcome the stress induced by the chocolate spotand its effect on the nutritional status of faba bean seed. The improvement of seed nitrogen content and crude

Table 5. The effect of single and dual application of antagonistic (*T. harzianum* AAUT14 and *B. subtilis* AAUB95) on yield related parameters, seed nitrogen and crude protein contents of faba bean (Trial 1-Ashebeka and Trial 2-Hachalu variety)

Treatments (Trial-1)	Parameters					
T1-Control (B.f only)	42.50^{c}	12096.7^{a}	3269.79^{e}	27^{c}	2.35^{c}	14.69^{c}
T2-*T. harzianum* AAUT14+B.f	58.17^{b}	10046.3^{b}	3764.58^{b}	38^{ab}	3.12^{ab}	20.00^{ab}
T3-*B. subtilis* AAUB95+B.f	48.67^{bc}	11355.7^{ab}	3542.51^{d}	31^{bc}	3.35^{ab}	20.94^{ab}
T4-*T. harzianum* AAUT14+*B. subtilis* AAUB95+B.f	69.00^{a}	10376.7^{b}	4391.45^{a}	42^{a}	4.00^{a}	25.00^{a}
T5- MORE 720 WP+B.f	56.33^{b}	11356.7^{ab}	3744.53^{b}	37^{ab}	2.5^{c}	15.63^{c}
T6- ORZEB 80WP+B.f	49.67^{bc}	10000.0^{b}	3660.32^{c}	33^{bc}	2.48^{c}	15.71^{c}
CV	22	0.10	0.13	0.16	0.33	0.34
Treatments (Trial-2)	**HSDW (g)**	**Biomass (kg/ha)**	**GYE (kg /ha)**	**HI (%)**	**SNC (%)**	**SCPC (%)**
T1-Control (B.f only)	43.50^{c}	12052.3^{a}	3170.83^{e}	26^{d}	2.32^{c}	14.50^{c}
T2-*T. harzianum* AAUT14+B.f	60.33^{b}	10079.7^{c}	3654.17^{b}	36^{b}	3.00^{ab}	19.00^{ab}
T3-*B. subtilis* AAUB95+B.f	49.33^{bc}	10063.3^{c}	3476.04^{d}	35^{b}	3.38^{ab}	21.13^{ab}
T4-*T. harzianum* AAUT14+*B. subtilis* AAUB95+B.f	68.00^{a}	11043.3^{b}	4378.12^{a}	41^{a}	3.75^{a}	24.00^{a}
T5- MORE 720 WP+B.f	57.17^{b}	10580.0^{bc}	3640.95^{b}	34^{b}	2.53^{c}	15.81^{c}
T6- ORZEB 80WP+B.f	50.00 ^{bc}	11020.0^{b}	3524.63^{c}	32^{c}	2.43^{c}	15.19^{c}
CV	0.24	0.10	0.16	0.13	0.33	0.32

B.f-Botrytis fabae, HSDW- Hundred seed dry weight, GYE/ha- Grain yield estimate per hectare, HI- Harvest index, SNC-Seed nitrogen content and SCPC- Seed crude protein content. Mean values of three replicas within same columns labeled with same letter (s) of superscript are not different (P=.05) according to DMRT and Tukey HSD Post Hoc analysis of Two-Way ANOVA

protein can be attributed to the fixation of nitrogen, phosphate solubilization and production of phytohormones [31]. Applying microbial consortium to plants is associated to increase the concentration of minerals such as nitrogen, phosphorous, potassium, magnesium, chlorophyll biosynthesis and photosynthetic activity that led to the accumulation of proteins and carbohydrates [32]. Nevertheless, the faba beans seed protein was reported to be 24-30%, the protein content of less 24% was detected in this study. This might be associated with the effects of the bio-agents and or the variation in the genotypes of the faba bean varieties used in this study. On the other side, the minimum seed protein content was seen in T1 of both varieties compared to the other treatments, that might be caused bythe effects of chocolate spot on the nutrient accumulation of faba bean seeds even after harvest.

4. CONCLUSION

In the present study, the mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 or in some cases *T. harzianum* AAUT14 performed best on faba beans growth and yield parameters as compared to the fungicides. The present study also demonstrated the future use of these antagonistic microorganisms for controlling chocolate spot of faba bean. Thus, this combination can be an input for faba bean production along with MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80WP (Mancozeb).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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