Evaluation of Rhizobacteria and *Trichoderma* isolates from the rhizosphere of faba bean (*Vicia faba* L.) for their Potential in the Management of Chocolate spot (*Botrytis fabae* Sard.) Disease and Host Plant Growth-Promoting Properties

A Dissertation Submitted

To

The school of Graduate Studies

Faculty of Life Sciences

Addis Ababa University

By

Zewdineh Firdu Weldeamlak

In partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Biology (Applied Microbiology).

February, 2021

Addis Ababa, Ethiopia.

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Supervisor: Dr. Fassil Assefa (Associate Professor)

Co-supervisor: Dr. Tesfaye Alemu (Associate Professor)

DECLARATION

I hereby declare that this dissertation entitled "**Evaluation of Rhizobacteria and *Trichoderma* isolates from the rhizosphere of faba bean (*Vicia faba* L.) for their Potential in the Management of Chocolate spot (*Botrytis fabae* Sard.) Disease and Host Plant Growth-Promoting Properties."** represents my own work which has been done after registration for the degree of PhD at Addis Ababa University and has not been previously included in a thesis or dissertation submitted to this or any other institutions for any other degree or professional qualifications.

Further, I have acknowledged all the sources used for this work and cited in the reference section.

Zewdineh Firdu Weldeamlak	GSR/1567/07	_____	_____
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Supervisor	Signature	Date

Dr. Tesfaye Alemu (Associate Professor)	_____	_____
Co-supervisor	Signature	Date

DEDICATION

This dissertation is dedicated to my late father, Firdu Weldeamlak, who passed away during the work of this research.

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I would like to express my sincere thanks to my supervisors, Dr. Fassil Assefa and Dr. Tesfaye Alemu, for their excellent advice, cooperation and for all the opportunity I was given to conduct my research under their supervision. Their expertise was invaluable in formulating the topic, organization, and write up of this dissertation. I would especially like to thank Dr. Fassil Assefa, as my teacher in both at the MSc and PhD level and as an advisor in this dissertation. He has shown me, what a good scientist and person should be.

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Zewdineh Firdu Weldeamlak

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LIST OF ABBREVIATIONS/ACRONYMS

BC-biocontrol

BCAs-biological control agents

BNF-biological nitrogen-fixation

B.f- *Botrytis fabae*

BSA- Bovine serine albumin

bv-biovar

CTAB- cetyl trimethyl ammonium bromide

DAI- days after inoculation

DAS-days after sowing

DoT- days of treatment

FAOSTAT-Food and Agriculture Organization Corporate Statistical Database

IDM- integrated disease management

RDW-root dry weight

R/SR-root to shoot ratio

SDW-shoot dry weight

TE-Tris ethylene diamine tetra acetic acid

Diversity of Rhizobacteria and *Trichoderma* isolates from the rhizosphere of faba bean (*Vicia faba* L.) for their Potential in the Management of Chocolate spot (*Botrytis fabae* Sard.) Disease and Host Plant Growth Promoting Properties

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ABSTRACT

Faba bean (*Vicia faba* L.) is one of the legume grains used as a source of food for human. However, the productivity of the crop is less due to chocolate spot (*Botrytis fabae* Sard) in Ethiopia. The disease can be managed through the applications of fungicides such as MORE 720 WP and ORZEB 80 WP. Alternatively, an eco-friendly approach is initiated using *Trichoderma* and bacterial species to manage the disease. Therefore, in this study, *Trichoderma* and bacterial species were evaluated against *Botrytis fabae* under *in vitro* and *in vivo* conditions. The data were analyzed by SPSS version 24. The bacterial strains inhibited *B. fabae* (6-68%) upon 3-9 days in the dual culture method. The highest inhibition was recorded by *B. tequilensis* AAUB100 that displayed 80% and *Serratia nematodiphila* AAUB146b with 79% inhibition at 20% (v/v) of the culture filtrate. A 200-760 µg mL^{-1} of solubilized phosphate was also released upon 3-6 days by the isolates. *Trichoderma harzianum* AAUT14 was the most effective antagonist with more than 88% of *B. fabae* inhibition in the dual culture upon 9 days. The isolates of *Trichoderma* also showed inorganic phosphate solubilization that ranged from 135-509 µg mL^{-1} upon 3-9 days. Under the greenhouse study conducted using a completely randomized design, the disease incidence reduced by 36-50% on Ashebeka variety compared to the control (T1) in which *T. harzianum* AAUT14 and *B. subtilis* AAUB95 (T4) displayed the highest reduction (50%) of disease incidence upon 60 days. The disease severity reduction was much pronounced upon 80 days ranging from 41-55% compared to the disease severity, which did not show significant difference ($p>0.05$) between T4 and T5 (MORE 720 WP) upon 60 days. A 3.8-34% of disease incidence and 15-26 disease severity reduction was also observed upon 60 days on Hachalu variety. An area under disease progress curve (AUDPC) of 405.97% and 415.33% was displayed by T5 and T6 (ORZEB 80 WP),

respectively on Ashebeka variety. Similarly, 377.98% by T5 and 412.48% by T6 of AUDPC was illustrated in Hachalu variety showing no significance difference ($p>0.05$) with T4. Under field conditions conducted using a completely randomized block design, the combination of *Trichoderma harzianum* AAUT14+*B.subtilis* AAUB95 (T4) reduced the disease incidence and severity showing no significance difference ($p>0.05$) with T5 upon 70 and 90 days. Nevertheless, in Hachalu variety (Trial 2), the treatment reduced the disease incidence from 23-51% and disease severity 37-54% upon 90 days. In addition, the AUDPC ranged from 1586.1-2250.0% in trial 1 and 1382.0-2454.5% in trial 2. The grain yield was also increased by 34% and 38% in trial 1 and 2, respectively over the controls by T4. In conclusion, the mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 or in some cases *T. harzianum* AAUT14 performed best in controlling chocolate spot and growth promotion of faba bean under greenhouse and field conditions.

Keywords: AUDPC; biocontrol; chocolate spot; disease severity; fungicides; inhibition; yield

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CHAPTER 1

1. Introduction

Faba bean (*Vicia faba* L.) is one of the grain legumes used as a source of food for human in developing countries and forage of animals in developed countries. This crop contains proteins (24-30%), carbohydrates (55%) and fat, 10% (GRDC, 2017). It is also known as broad bean, horse and field bean which is the fourth most widely grown cool season legume after pea, chickpea and lentil in the world (FAOSTAT, 2019). In Ethiopia, it is mainly grown in the mid and high altitude areas having 1800-3000 meters above sea level (Gemechu Kenneni *et al*., 2006), and the country is the second most important faba bean growing country next to China. Farmers integrate the crop in rotation with cereals to improve soil fertility and enhance production. In addition, the straw of faba bean is used to make brick and as a fuel in some parts of the country.

In spite of its importance, the productivity of faba bean is about 1.8t ha^{-1}, which is far from the crop's potential of more than 5t ha^{-1} due to several biotic and abiotic factors (Maalouf *et al*., 2016). From biotic factors, faba bean is attacked by more than 100 diseases caused by microbial pathogens such as fungi, bacteria, nematodes and viruses that severely affect the productivity of the crop (Hebblethwaite, 1983). In Ethiopia, fungal disease such as chocolate spot (*Botrytis fabae*/*cinerea*), rust (*Uromyces viciae fabae*), black root rot (*Fusarium solani*), ascochyta blight (*Ascochyta fabae*) and fusarium root rot (*Fusarium avenaceum*) are implicated with low productivity of faba bean (Shiferaw Deneke, 2018).

Plant diseases can be managed through the application of pesticides, crop rotation, proper sanitation and storage. Nevertheless, the use of pesticides can result in environmental pollution, as residues on food, toxicity and the pathogens develop resistance to the chemicals following repeated applications and needs eco-friendly options (Soni *et al*., 2007). The application of phytobeneficial microbes has drawn attention as part of integrated pest management (IPM) for better plant growth promotion and yield (Glare *et al*., 2012). Amongst the microorganisms, antagonistic rhizospheric soil fungi like *Trichoderma* species and bacteria such as *Bacillus* and *Pseudomonas* species, have received much attention as biological control agents (BCA's) against different plant diseases. The

persistence of BCAs in the soil, particularly on the rhizosphere, eventually associated as endophytes, would guarantee their long-term advantages in plants (Woo *et al*., 2014).

Trichoderma species are soil borne fungi that are endowed with a variety of mechanisms to control plant pathogens and enhance growth promotion of plants. *Trichoderma* species depend on mycoparasitism as a means of biocontrol agents and produce different types of metabolites and solubilize insoluble inorganic phosphate to enhance plant growth. Rhizosphere soil bacteria such as *Bacillus subtilis* and *Pseudomonas fluorescens* are also capable of promoting plant growth and suppress pathogens. Rhizosphere soil bacteria together with fungi can be utilized as growth promoters of plants and antagonists against pathogens to protect host plants from disease infection (Nourozian *et al*., 2006).

Chocolate spot (*Botrytis fabae/cinerea*) is one of the major fungal diseases affecting faba bean production in central Ethiopia. It is widely expanding to different parts of the country compared with fungal diseases such as ascochyta blight, faba bean rust and root rot (Dagne Kora *et al*., 2017). The disease infects all the aerial parts and the symptom can be seen as small reddish-brown circular or oval spots on stems, leaves, flowers, seeds of the faba beans and the disease can decrease seed quality (GRDC, 2017). Once the disease is established, it rapidly spreads within the crop, most aggressively under warm and humid conditions. It is favored by a temperature that range from 15-25°C and relative humidity of >70% extending for 4-5 days. The yield loss caused by the epidemics of chocolate spot has been reported to reach as high as 50-100% on highly susceptible faba bean varieties in countries such as Australia, Egypt, England and China (Saber *et al*., 2011; Abo-Hegazy *et al*., 2012). In Ethiopia, the yield losses of up to 67.5 % was recorded in susceptible faba bean cultivars by chocolate spot disease (Samuel Sahile *et al.*, 2010).

Farmers use chemicals such as MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80WP (Mancozeb) for the management of fungal diseases. However, attention has been given to manage fungal diseases of faba bean in Ethiopia and other countries using BCAs (Pandey and Chandel, 2014). To that end, attempts have been made to use BCAs in crops disease management including chocolate spot disease in Ethiopia. Consequently, different studies were conducted under *in vitro* and *in vivo* conditions to manage crops diseases (Sharga, 1997; Samuel Sahile *et al*., 2009; El-Banoby *et al*., 2013; Barakat *et al*., 2014; Fekadu Alemu, 2016).

Most of the effective antagonistic studies were obtained from the fungal species, mainly *Trichoderma* spp. where the majority of BCA reports from bacteria revolved around *Bacillus* and *Pseudomonas* spp. The antagonistic *Trichoderma* spp. (Ermias Teshome *et al*., 2013) and *T. album* (Barakat *et al*., 2014) reduced the disease incidence of chocolate spot by 78% and 51%, respectively. Mbazia et al. (2016) have also showed 35% rate of chocolate spot infestation reduction on leaves of faba beans by *T. viride*. Likewise, bacterial isolates; *Bacillus macerans* (Sharga, 1997), and co-inoculated with *P. fluorescens* and *B. subtilis* (El-Banoby *et al*., 2013) reduced the disease incidence of chocolate spot by 33% and 58%, respectively. Fekadu Alemu (2016) has showed that *P. fluorescens* isolates showed 88% mycelial growth inhibition of *B. fabae* under *in vitro* conditions. Samuel Sahile et al. (2009) have showed that isolates of *Bacillus* spp. reduced the growth of *B. fabae* (chocolate spot) to the range of 23-64% under *in vitro* study.

In Ethiopia, Arsi and Bale zones are the major faba growing areas and at present time, the symptom of chocolate spot is widely seen on faba bean crops in Eastern Arsi at Digeluna Tijo, Tiyo woreda and in Bale zone of Goba woreda. (Dagne Kora *et al*., 2017; Shiferaw Deneke *et al*., 2018). Therefore; this study was initiated to study on screening of effective fungal (*Trichoderma* spp.) and bacterial antagonists to control *B. fabae* (chocolate spot) and promote growth of faba bean under greenhouse and field conditions.

1.2 Objectives and Assumption of the Study

1.2.1 General Objective

The general objective of this study was to evaluate the potential of selected fungal (*Trichoderma* spp.) and bacterial antagonists as biological control agents of chocolate spot (*Botrytis fabae*) disease and to determine their growth-promoting properties of faba bean (*Vicia faba* L.) under greenhouse and field conditions.

1.2.2 Specific Objectives

The specific objectives of this study were to:

i. Isolate and characterize antagonistic bacteria from the rhizosphere of faba bean (*Vicia faba* L.) for their antagonistic potential against *Botrytis fabae* and multiple plant growth-promoting properties of the host under *in vitro* conditions.
ii. Isolate and characterize potential *Trichoderma* isolates from the rhizosphere of faba bean (*Vicia faba* L.) and for their antagonistic effect against *Botrytis fabae* and plant growth-promotion under *in vitro* conditions.
iii. Evaluate the effects of selected *Trichoderma* sp. and bacterial antagonist (*Bacillus* sp.) on the control of chocolate spot and growth promotion of faba bean (*Vicia faba* L.) performance under greenhouse conditions.
iv. Evaluate the field performance of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 on faba bean (*Vicia faba* L.) growth promotion and management of chocolate spot under naturally infected field conditions.

1.2.3 Study Assumption

The assumption of this study was stated as: Plant growth promoting rhizospheric soil bacteria (*Bacillus* sp.) and fungi (*Trichoderma* spp.) will suppress the occurrence of foliar fungal disease, chocolate spot, caused by *Botrytis fabae* and promote growth of the host plants (faba beans). Thus, this study would create an awareness to an alternative strategy of chocolate spot disease management and growth promotion of the host plant.

1.3 LITERATURE REVIEW

1.3.1 Faba bean (*Vicia faba* L.)

Faba bean (*Vicia faba* L.) which is also known as broad bean, horse bean or field bean is the fourth most important cool season food legume in the world (FAOSTAT, 2019). It is one of the major food and feed leguminous crop which is rich in protein and starch. As a result, it is most commonly included in the diets of inhabitants of the Middle East, the Mediterranean region, China and Ethiopia (Asfaw Tilaye *et al*., 1994). Ethiopia is considered as the secondary center of diversity of faba bean and the crop plays a major role in the diet of the population.

The crop is grown as a field crop throughout the high lands and is most common in Wayena Dega or temperate zone and Dega at an altitude ranging from1800-3000m above sea level and receiving an annual rainfall of 700-1100 mm (Gemechu Keneni *et al*., 2006). The crop is widely cultivated as a source of human food and animal feed. In Oromia and Amhara region about 437,106.04 hectares of lands with the annual production of 9, 217, 615.35 and 21.09 quintals/ha (CSA, 2018). Although the majority of faba bean produced is consumed domestically, the smallest percentage of the crop is delivered to the export market and put Ethiopia among the top broad bean exporting countries next to China (CSA, 2018). This days, it is one of the most common crops that is under production. However, the productivity of faba bean in Ethiopia is still far from its potential due to biotic and biotic factors (Tamene Temesgen *et al.,* 2015).

Moreover, faba bean is one of the pulse crops that is used as green manure and crop rotation in low inputs agricultural systems to supply nitrogen to subsequent crops, and maintain soil fertility in the cereal cropping systems of the Ethiopian highlands (Amanuel Gorfu and Tanner, 1991). This is mainly because faba bean fixes atmospheric nitrogen, biological nitrogen-fixation (BNF) by its symbiotic relationships with soil bacterial species known as rhizobia. According to Somesagaran and Hoben (1994), faba bean is one of the highest nitrogen fixing crops with an average of 240-325kg N ha^{-1} yr^{-1}.

1.3.2 Fungal Diseases of Faba bean

Studies have shown that fungal diseases such as chocolate spot, cercospora leaf spot, downy mildew, rust, ascochyta blight, gall, fusarium root rot, anthracnose, alternaria leaf spot, sclerotinia stem rot, aphanomyces, pythium and rhizoctonia root rot, stemphylium blight and powdery mildew are affecting faba bean in Ethiopia (Samuel Sahile *et al*., 2009; 2011; Ermias Teshome *et al*., 2013; Dagne Kora *et al*., 2017). However, yield loss caused by these diseases may vary from area to area based on the susceptibility of faba bean varieties and edapho-climatic conditions of the area. The yield loss associated with some fungal disease of faba bean is summarized in Table 1.1.

Table 1.1: Economically important foliar, non-foliar fungal diseases, causal agents (pathogens) and the associated yield loss of faba bean (*Vicia faba* L.)

Disease	Pathogen	Status or Yield loss (%)	Reference (s)
Alternaria leaf spot	*A. alternata*	Minor disease	GRDC (2017)
Anthracnose	*C. truncatum*	>5	Ondrej and Hunday (2007)
Chocolate spot	*B. fabae/cinerea*	67.5	Samuel Sahile et al. (2010)
		50-100	Abo-Hegazy *et al*., 2012
			Saber *et al*., 2011
Ascochyta blight	*A. fabae*	30-50	GRDC (2017)
		35-40	Hanounik and Robertson (1989)
Rust	*U. fabae*	30.00	GRDC (2017)
		<20	Multari et al (2015)
Stem phylium blight	*S. botryosum*	Minor disease	GRDC (2017)

1.3.3 Faba bean Production and Fungal Diseases of Faba bean in Ethiopia

Faba bean (*Vicia faba* L.) is the first legume that covers large area of coverage and production in Ethiopia of other legume crops. According to CSA (2018) Faba bean is being grown on 437,106.04 hectares of lands with the annual production of 9, 217, 615.35 and 21.09 quintals/ha in Ethiopia. It is one of the most common crop that is under production in Oromia and Amhara region. the area that is used for the production of faba bean covers 204, 387.86 hectares with total production of 4, 832, 016.57 and 23.64 quintals/ha in Oromia region and 150, 934.92 hectares with total production of 2, 836, 912.59 and 18.80 quintals/ha in Amhara region.

However, the production faba bean is being influenced by several biotic and abiotic constraints in the country. From biotic constraints several fungal diseases are reported economically important in faba bean production. Disease such as chocolate spot (*Botrytis fabae/cinerea*), rust (*Uromyces viciae fabae*), black root rot (*Fusarium solani*), ascochyta blight (*Ascochyta fabae*) and fusarium root rot (*Fusarium avenaceum*) are economically important in contributing to the low productivity of faba beans (Shiferaw Deneke, 2018; Fikiru Wakoya, 2020). Of these diseases chocolate spot is the most prevalent and its associated yield loss is reported by several researchers. In this aspect a yield loss that varied from 34% on tolerant genotypes up to 61% in susceptible genotype and Bouhassan et al. (2003) have reported a 60-80% of faba bean yield loss due to chocolate spot in Ethiopia.

1.3.4 Faba bean Production and Chocolate Spot Disease of Faba bean in Arsi and Bale Zones

According to CSA (2016) in Arsi zone about 31, 360.11 hectare is being used for faba bean production with 764, 923.96 quintals, while 16,471.36 hectares is faba bean arable land and producing 388, 302.53 quintals in Bale zone. Faba bean (*Vicia faba* L.) like other leguminous and non-leguminous crops is being affected by fungal disease and that can limit yields through time. The prevalence of chocolate spot (*Botrytis fabae*/*cinerea*) disease is being observed in Bale and Arsi zones this day (Figure 1.1). Chocolate spot (*Botrytis fabae*/*cinerea*), ascochyta blight (*Ascochyta fabae*) and faba bean rust (*Uromyces fabae*) are the major fungal diseases affecting

faba bean production in central Ethiopia, Arsi and Bale Zones. In Bale zone the yield loss by chocolate spot is reported to be 29.1-47.8% in different faba bean varieties (Dagne Kora *et al*., 2017).

(a) (b)

Figure 1.1. Symptoms of chocolate spot caused by *B. fabae* on faba bean in Bale (a) and Arsi zone (b) (photos were taken during sampling time, October, 2016).

1.3.5 Distribution and Symptoms of Chocolate Spot

Chocolate spot is caused by pathogenic fungi *Botrytis fabae/cinerea* that infects the faba bean crops. This pathogen survives by producing resistant structure (sclerotia) on, or in, crop debris and seeds. Having sclerotia, under suitable conditions, conidia are produced and presumably dispersed by wind and rain. Chocolate spot is widely distributed in Tunisia, Algeria, Morocco, Libya, Ethiopia and other countries such as Spain, Norway, Germany, Scotland, Russia, Japan, China, Canada and Australia (Hebblethwaite, 1983).

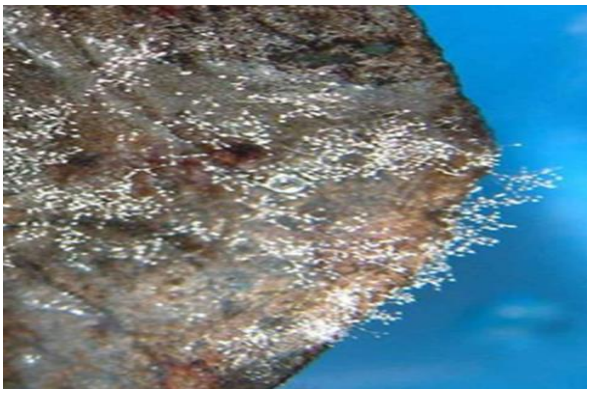
The symptom of chocolate spot is visible on the aerial part of infected faba bean leaves, pods, stem and flower as small reddish-brown circular or oval spots. The spots on leaves and stems enlarge

and develop a grey, dead center with a red-brown margin. The conidial lengths of chocolate spot caused by *B. fabae* are found to be 12.7-22.4 x 9.4-17.8 micron with an ovoid- elliptic shape; sclerot lengths are found to be 1.0-4.9 x 0.3-3.2mm with irregular shape and black color (Colak ates *et al*., 2017). Often, two stages of the disease have been observed. The non-aggressive stage known by the small circular and discrete reddish spots with darker margins visible on one side of leaf and the aggressive stage where the small spots merge and coalesce to form irregular larger dark-brown lesions involving the entire leaf surface.

Under favorable conditions the disease spreads quickly and causes severe defoliation, flower drop, stem collapse, tissue necrosis and finally death (Singh *et al*., 2018). At storage, *B. fabae* causes decrease in seed quality through decolorization and these have significant impact on market price of the product. Small, black sclerotia can sometimes be found inside the stems of badly diseased plants. According to GRDC (2017) under moist conditions, the fruiting structures may be visible, protruding as grey hair-like formations of spores from the underside of diseased leaves (Figure 1.2).

(a)

(b)



(c)

(d)

(e)

(f)

Figure 1.2: Infections of chocolate spot in faba beans start as small brown spots (a), chocolate spot lesion in the field, showing some expansion across the leaf surface (b), chocolate spot showing lesion expansion and sporulation after a few days in humid chamber (c), chocolate spot infecting flowers set (d), chocolate spot lesion on pod leading to infection and staining of the inside seed (e) and Stained faba beans seeds under storage (f) (*Source: GRDC, 2017*).

2. Control of Fungal Diseases in Faba bean

2.1 Chemical Control (CC)

Fungal disease of faba bean can be controlled through the application of fungicides as means of combating plant pathogens. Chemicals such as MORE 720 WP (Mancozeb+Cymoxanil), ORZEB 80 WP (Mancozeb), Carbendazim, Chlorothalonil, and Procymidone can be used for the management of chocolate spot and rust and Azoxystrobin (Ortiva) for the management of ascochyta blight. The recommended amount for specified disease type, application rate and withholding period of these fungicides (Kabbabeh *et al*., 2009).

Table 1.2: Fungicides used for foliar disease management of faba bean (Kabbabeh *et al*., 2009; Zenith Crop Sciences, 2020)

Fungicide	Amount	Disease Type	Application Rate	Withholding Period
Orzeb 80WP	420g/L	Chocolate spot	3kg/ha	Harvest: 1 day
More 720 WP	500g/L	Chocolate spot	2kg/ha	Harvest: 7 days
		Chocolate spot	1.7-2.5kg/ha	Harvest: 7 days
Mancozeb	750g/L	Ascochyta blight		
		rust	1.7-2.2kg/ha	
	500g/L	Chocolate spot	500g/ha	Harvest: 30 days
Carbendazim	500g/L	Chocolate spot	500mL/ha	
Chlorothalonil	720g/L	Chocolate spot	1.4-2.3/ha	Harvest: 7 days
		Rust		
Procymidone	500g/L	Chocolate spot	0.5L/ha	Harvest: Nil
Copper	500g/L	Chocolate spot	4.6L/ha	Harvest: 1 day
		Rust		

2.1.1 MORE 720 WP, ORZEB 80 WP and their Mode of Actions

According to Zenith Crop Sciences (2020), MORE 720 WP is a systemic and contact fungicide used as a foliar spray to provide both protective and curative action against late blight in potato crops. It consists Mancozeb and Cymoxanil as active ingredients. The combination of this mixture as MORE 720 WP is effective when used in plant protection system from pathogenic fungi of different crops such as rice, mango, and other fruit trees, vegetables, potato, tobacco, and other agriculturally important crops such as faba bean and other legumes. The unique mode of actions of Cymoxanil is that it helps to penetrate into the leaf tissue and act on the emerging target pathogens.

ORZEB 80 WP is also an agriculturally important fungicide that contains only Mancozeb as active ingredient. This ingredients, Mancozeb, forms a protective film and can prevent the plants from a penetration of the future infections. The chemical group of Mancozeb is Dithiocarbamate and Cyanoacetamide oxime is the chemical group of Cymoxanil. Both MORE 720 WP and ORZEB 80WP are wettable powder in their formulation and applied at a rate of 2 and 3kg/ha, respectively, upon appropriate weather conditions (when it is not raining and the temperature is not above 28-30 °C (Zenith Crop Sciences, 2020).

2.2 Biological Control (BC)

Biological control (BC) is also another tool of pest and disease management of different crops (Deshmukh *et al*., 2010). In this context, soil microorganisms such as bacteria and fungi are used as biopesticides. Fungi such as *Trichoderma* and PGPR, especially *Bacillus* and *Pseudomonas* are well studied in reducing different fungal diseases of several crops. In this regard, the progress in research of certain biological control agents in disease management of some faba bean fungal diseases (Table 1.3).

Table 1.3: Fungal diseases of faba bean and their management using biological control agents

Name of Disease	BCAs	% Reduction	Reference
Chocolate spot	*T. album*	77.78	Barakat et al. (2014)
	B. macerans BS153	33.28	Sharga (1997)
	Trichoderma spp.	42.22-51.11	Ermias Teshome et al. (2013)
	P. fluorescens and *B. subtilis*	>57.40	El-Banoby et al. (2013)
Fusarium root rot	*T. viride*	67.00	Eshetu Belete et al. (2015)
	Bacillus spp.	39.00-44.00	Eshetu Belete et al. (2015)
	T. viride	55.58	Abdel-Monaim et al. (2013)
	T. harzianum	57.30	Abdel-Kader et al. (2015)
	B. subtilis	63.60	Abdel-Kader et al. (2015)
Fusarium wilt	*T. viride*	44.69	Abdel-Monaim et al. (2013)
Alternaria leaf spot	*B. megaterium*	40.23	Abdel-Monaim et al. (2013)
	B. subtilis	89.00	Behairy et al. (2014)
	T. harzianum	79.00	Behairy et al. (2014)

2.3 Integrated Disease Management (IDM)

Integrated disease management (IDM), that combines biological, cultural, physical and chemical control strategies in a holistic way rather than using a single component strategy proved to be more effective and sustainable (El Khoury and Makkouk, 2010**)**. According to Pande et al. (2009) IDM strategy for controlling foliar diseases of faba bean includes the use of disease free seed, avoid sowing too early to minimize disease, follow 3 to 4 years crop rotation and select the tolerant variety to the main disease risk in particular region. Thorough and regular crop monitoring is essential if the strategic spray program is to be successful. The timing of fungicide applications depends on the disease level observed, the time since the previous application, and the likelihood of rainfall and other conditions conducive to infection and spread of chocolate spot. Carbendazim is best used when high chocolate spot pressure occurs or when rapid plant growth produces large amounts of unprotected foliage, particularly from mid-flowering onwards. Chlorothalonil and Mancozeb are best to be used earlier if rust or Ascochyta is a problem.

On the other hand, seed treatment with P-pickle T1(Thiram and Thiabendazole) is recommended for *Pythium* and *Fusarium* management of faba bean and also for *Ascochyta* and *Botrytis* spp (Hawthrone *et al*., 2004). Apart from conventional fungicides, the oil of basil (*Ocimum basilicum*) possesses antifungal properties against *B. fabae* of infected faba bean seedlings (Oxenham *et al*., 2005). Application of salicylic, benzoic, citric and oxalic acids and ribavirin enhanced the resistance of faba bean infected by *B. fabae* (Hassan *et al*., 2006). According to Barakat et al. (2014) spraying of faba bean plants with *Trichoderma* spp. and Bio-Zeid as a biofungicide, before inoculation with *B. fabae* significantly reduced chocolate spot disease severity. The severity of the disease 14 days after inoculation with *Trichoderma* spp. was in the range of 3.0-4.9%, compared with Bio-Zied and control, being 3.00 and 8.70% respectively.

2.4 Other Practices

Other practices are methods that reduce disease establishment, reproduction, dispersal and survival in a habitat. For example, changing irrigation practices can reduce pest problems, since too much water can increase root diseases in plants, it negatively impacts rooting ability to absorb nutrients due to suffocation causing plant death and total crop failure (Rajanna *et al*., 2018). On the other

hand, crop rotation helps to prevent soil-borne diseases. If crops are grown in the same place each year, it is inevitable that the chance for the occurrence of pest and diseases become much greater. For example, the minimum time interval that is needed is four years for chocolate spot, ascochyta blight, rust and cercospora leaf spot (Richardson, 2008). Yet longer periods may be required for powdery mildew, since oospores can survive at significant levels for at least 10 years in the pre-infected field soil (Biddle, 2001). Proper seed storage and use of disease free seeds for sowing is also essential for the management of seed-borne diseases of several crops.

2.5 Biological control agents (BCAs) and Their Mechanism of Actions

Plant diseases can be managed through the application of fungicides, crop rotation, using disease free and disease resistance seeds. Nevertheless, the use of pesticides can result in environmental pollution, residues on food, toxicity and the pathogen develops resistance to chemicals following repeated application (Soni *et al.,* 2007). Biological control (BC) via the application of BCAs has drawn attention as part of integrated disease management (IDM) for the better promotion of plant growth, yield and yield related parameters (Glare *et al*., 2012).

Biocontrol agents (BCAs) are defined as a means of reduction in the amount of inoculum or disease producing ability of plant pathogens and pests through the application of one or more microorganisms (Pal and Gardener, 2006). The concept of BCAs was initiated in the 1920's from the observation where the introduction of antibiotic producing microbes suppressed plant pathogens. The application of antagonistic rhizospheric soil fungi like *Trichoderma* species and bacteria such as *Bacillus* and *Pseudomonas* species have received much attention for biological control of pests and diseases. Biological control agents are endowed with different direct, indirect mechanisms by which they can antagonize the pathogens (Table 1.4).

Table 1.4: Type of antagonism, mechanisms and associated example of biological control agents (Pal and Gardener, 2006)

Type	Mechanisms	Example
Direct antagonism	Mycoparasitisim	Lytic and non-lytic mycoviruses *Ampelomyces quisqualis* *Lysobacter enzymogenes* *Pasteuria penetrans* *Trichoderma virens*
	Antibiotics	2,4-diacetylphloroglucinol Phenazines Cyclic lipopeptides
	Lytic enzymes	Chitinases Glucanases Proteases
Mixed-path antagonism	Unregulated waste products	Ammonia Carbon dioxide Hydrogen cyanide
	Physical/Chemical interference	Blockage of soil pores, germination signals consumption, molecular cross-talk
	Competition	Exudates (leachates consumption) Siderophore scavenging Physical niche occupation
Indirect antagonism	Induction of host resistance	Contact with fungal cell walls Detection of pathogen-associated, molecular patterns Phytohormones-mediated induction

2.6 Fungi as Biocontrol and Growth Promoters of Plants

2.6.1 *Trichoderma* species as Biocontrol Agents

Trichoderma species are among the most frequently isolated fungi and are present in all the root ecosystems. The fungi are the plant symbionts that function as parasites and antagonists of plant pathogenic fungi. *Trichoderma* species are cosmopolitan fungi that are present in all types of soil and other habitats (wood decay, barks etc.). *Trichoderma harzianum*, *T. koningii*, *T. orientale*, *T. tomentosum* and *T. viride* are usually found in cellulose rich soil and other environments (Jang *et al*., 2017). *Trichoderma* species can grow very quickly within 24 to 48 hrs. and can easily compete and over grow other fungi. *Trichoderma* species are well effective in controlling phytopathogens due to their ability to grow toward the hyphae of other fungi, coil around them and degrade the cell walls of the pathogen.

Induced systemic resistance (ISR) and lytic enzymes are involved in both antagonistic and saprophytic processes providing an advantage to biodegrading and antagonistic potential, for the efficient colonization of different ecological niches in soil. A principal role in mycoparasitism is attributed to the synthesis of enzymes such as chitinases, glucanases and protease. Proteases are also significantly involved in cell wall degradation, since fungal cell walls contain chitin and glucan polymers embedded in and covalently linked to a protein matrix (Kapteyn *et al*., 1996). The antagonistic potential of *T. harzianum* and *T. viride* was studied against wilt and wet rot in plants. The antagonism was also noticed by the application of *T. harzianum* and *P. fluorescens* against *Helminthosporium* infection in *Chrysalido carpus* (Jegathambigai *et al*., 2009), which points the induction of resistance mechanisms as hypersensitive response (HR), systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants. The antagonistic activity of *Trichoderma* spp. against various soil born and foliar pathogens viz., *Sclerotium rolfsii*, *Colletotrichum gloeosporioides*, *C. capsici*, *R. solani*, *B. cinerea*, *Colletotrichum* spp., *Magnaporthe grisea*, *Phytophthora* spp., *Alternaria* spp. etc.), bacteria (*Xanthomonas* spp., *Pseudomonas syringae*, etc.) as well as viruses (cucumber mosaic virus) was reported (Khang *et al*., 2013).

Trichoderma strains, viz; *T. harzianum*, *T. viride* and *T. virens* are the ones that have been well studied and identified as having potential applications in biological control of fungal disease in greenhouse and field studies. The efficacy of the *Trichoderma* isolates ranges from 47.6 to 98% in controlling chocolate spot of faba bean (Samuel Sahile *et al*., 2011). Mbazia et al. (2016) have reported 35% rate of chocolate spot infestation reduction on leaves of faba beans by *T. viride*. The dual culture and volatile metabolites assays showed that *T. harzianum* suppressing the growth of *Fusarium solani* by 51.4 and 38.1%, respectively and tomato plants treated by the same strain showed 117.5 and 138.9% increase in plant height and dry weight, respectively and the disease incidence reduction of 55.5% compared to the control (Bokhari and Perveen, 2012). Another study showed that the number of wilted chickpea plants infected with *F. oxysporium* was reduced by 67.93% due to inoculation with *T. harzianum* (Subhani *et al*., 2013). *Trichoderma harzianum* that showed antagonistic activity against *F. oxysporum* and *R. solani* resulted in 65%, 70%, and 58% increase of shoot length, root length and root dry weight of chickpea, respectively, compared to control (Yadav *et al*., 2011).

Quiroga-Rojas et al. (2012) have showed that a wild isolate of *Trichoderma* species and a commercial product of *T. lignorum* inhibited the mycelial growth of *Fusarium* species at 42 and 93.6%, respectively. *Trichoderma atroviride* reduced 27-36% of the known potato disease, late blight caused by *P. infestans* (Al-Mughrabi, 2008). Farah and Nasreen (2013) have evaluated *T. viride* and *T. harzianum* for their antagonistic activity against five different pathogens *in vitro* viz, *Colletotrichum lindemuthianum, F. oxysporum, R. solani, A. solani,* and *F. solani*, where the antagonists reduced 63.89-67.45% mycelial growth of the test pathogens. The biocontrol potential of two *Trichoderma* species on sclerotia rot disease of tomato showed that, *T. harzianum* and *T. viride* reduced the disease by 74.50%, and 68.75%, respectively (Kator *et al*., 2015).

2.7 Biocontrol Properties (mechanism of suppression) of the *Trichoderma* Isolates

2.7.1 Mycoparasitisim

Mycoparasitisim is the direct attack of one fungus on another; it is a complex process that involves sequential events of recognition, attachment, penetration and killing of the associated pathogens. In this process, the fungi secret cell walls degrading enzymes (CWDEs) that hydrolyze the cell wall of the pathogen. Once the fungi come into contact, *Trichoderma* attach to the host, coil around it and form appressoria on the surface of the pathogens. Attachment is mediated by the binding of carbohydrates in the *Trichoderma* cell wall to the lectins of the target fungi. Sonkar (2019) has observed the interaction between *Trichoderma* species and *F. oxysporum*, the sequence of events is categorized as pre-contact antagonistic interaction, chemo-attractive intermediate phase and finally parasitic interaction. Several studies have reported that most isolates of the genus *Trichoderma* to act as mycoparasites of many economically important faba bean fungal pathogens. For example, *Trichoderma harzianum* Rifai (Soliman *et al*., 2016), and *Trichoderma reesei* (Magdy *et al*., 2008) mycoparasitized *B. fabae*

2.7.2 Antibiosis and Competition for Nutrients

Trichoderma species are known to produce a plethora of secondary metabolites with inhibitory activity against diverse group of pathogenic microorganisms. The production of secondary metabolites in *Trichoderma* strain dependents on the antimicrobial substances belonging to different chemical classes. One of the best studied secondary metabolites from the biocontrol perspective is the ‘coconut aroma’ volatile pyrone 6-pentyl-2H-pyran-2- pyrone, commonly produced by *Trichoderma* species (Reino *et al*., 2008).

The existence of sufficient nutrient is essential for the growth and survival of microbes. Thus, nutrient limitation results in reduced conidial germination and germ tube development of the pathogens, thereby reducing the number of infection sites and the extent of subsequent necrosis caused by the pathogen. The pathogens enter into the plant body through wounds, senescing tissues

and natural openings (stomata and lenticels). However, *Trichoderma* aggressively colonize the points of entry and prevents the introduction of pathogens into host as confirmed in *B. cinerea* where the infection was prevented by 25% in straw berry treatment (Card *et al*., 2009).

2.7.3 Volatile and Non-volatile Secondary Metabolites ($NVSM_S$) Production

These are low molecular weight and non-polar compounds. Volatile metabolites comprise diverse group of organic compounds, generally with a molecular weight in the range of 50-200 Daltons. The production of many volatile metabolites like pyrones and sesquiterpenes have been reported as the mechanisms of *Trichoderma* species used to control pathogens. *Trichoderma* spp. are known to produce volatile secondary metabolites that are effective in the control of pathogens. For example, *T. harzianum, T. hamatum, T. asperellum* and *T. atroviride* are commercialized for the control of phytopathogens and plant growth promoters in agriculture. Non-volatile Secondary metabolites are known inhibitors of pathogens. These metabolic products are produced by different groups of fungal communities. The productions of $NVSM_S$ have been reported to have an important role in the biological control mechanisms of *Trichoderma* spp. Non-volatile secondary metabolites such as peptaibols produced by *Trichoderma* spp. are well investigated (Reino *et al*., 2008).

2.8 Rhizosphere Bacteria as Biocontrol Agents

Soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues and stimulate plant growth are collectively known as plant growth promoting rhizobacteria (Vessey, 2003). These useful microorganisms are used with the aim of improving crop yields by augmenting nutrient availability, enhancing plant growth and protection of plants from diseases and pests. The genera commonly used as bioprotectants of plants from diseases are *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium*, *Streptomyces*, etc.

Among PGPR, *Pseudomonas* and *Bacillus* species play crucial role in controlling fungal plant pathogens. *P. aeruginosa* strains inhibited the growth of *S. sclerotiorum* by 62-83% (Deshwal, 2012), *P. fluorescens* inhibited the radial growth of *P. infestans* under greenhouse conditions up

to 88% in potato tuber, *T. viride* and *P. infestans* showed a radial pathogen growth inhibition of 36.7% with complete overgrowth of *T. viride* on *P. infestans* (Ephrem Debebe *et al*., 2011). The antagonistic study conducted using *P. aeruginosa* significantly reduced the incidence of fusarium wilt disease of eggplant up to 85% (Altinok *et al*., 2016), *P. fluorescens* isolates showed 88% mycelial growth inhibition of *B. fabae* (Fekadu Alemu, 2016). El-Banoby et al. (2013) reported the BC activity of *P. fluorescens* and *B. subtilis* against chocolate spot reduced the diseases severity more than 57.4% in field conditions.

Bacillus is the dominant gram positive bacteria that is widely distributed in the soil and plant rhizosphere region. Ajilogba et al. (2013) studied the antagonistic effects of *Bacillus* species as biocontrol tools of tomato fusarium wilt under *in vitro* and *in vivo* study. They showed that *B. amyloliquefaciens* displayed 95.2% growth inhibition on *F. solani*, while *B. cereus* showed growth inhibition of 55.7%. In the same study, *B. pumilus* and *B. subtilis* showed inhibition of 70.46% and 82%, respectively. However, under *in vivo* study *B. cereus* showed disease incidence and disease control by 18.75% and 81.2%, respectively. Mangalanayaki and Durga (2016) reported that *B. pumilus* and *B. subtilis* controlled the growth of *F. solani* by 95.20% and 30.50%, respectively. *Bacillus* spp. also displayed 23-64% inhibition (Samuel Sahile *et al*., 2009), Ahmed (2015) and El-Banoby et al. (2013) have reported the inhibitory activity of *Bacillus subtilis on* the mycelial growth *B. fabae*, by 62.6% and 67.03%, respectively.

De Senna et al. (2017) showed that *S. plymuthica* inhibited the growth of *Botrytis cinerea* by 51%; whereas Ahmed (2016) illustrated *S. marcescens* inhibited *R. solani* by 54.81%. The culture filtrate of *S. marcescens* suppressed the mycelial growth of *R. solani* by 65.6%, through the application of 100 µL (El Khaldi *et al*., 2015). On the other hand, Aydi-Ben Abdallah et al. (2017) screened for antifungal activity of culture filtrate of *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycoperscisi* (FOL), and showed a decrease of radial growth by19.52% under *in vitro* conditions.

2.9 Growth Promotion and Biocontrol Mechanisms of Rhizospheric Soil Bacteria

2.9.1 Indole-3-Acetic Acid (IAA) and Ammonia Production

Indole-3-acetic acid (IAA) is one type of plant hormone which is the most important and native form of auxin in nature. It acts as a signal molecule for the regulation of organogenesis, tropic responses, cell expansion, division and differentiation in plant development (Ryu and Patten, 2008). Plant growth-promoting rhizobacterial isolates such as *P. aeruginosa*, *P. putida, B. subtilis, P. polymyxa* and *B. boronophilus* are capable of producing IAA. Kharwar et al. (2007) have showed the dual function of *P. fluorescens* which not only inhibited the mycelial growth of *Macrophomina phaseolina* under in vitro condition, but also produced IAA. The production of IAA can alter the metabolism and morphology of plants, leading to better absorption of minerals and water, giving rise to larger and healthier plants (DE-Bashan *et al*., 2008). Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils.

It has been reported that ammonia production indirectly influences plant growth and development. To this effect, several *Bacillus* and *Pseudomonas* spp. are efficient in the secretion of ammonia and significantly increased the biomass of medicinal and aromatic plant. Over 90% of the isolates from the rhizosphere of agricultural crops produced ammonia (Joseph *et al*., 2007). Isolates of *Pseudomonas* sp. PGPR-2 and PGPR-3 produced ammonia and improved seed germination in chickpea (Kaur and Sharma, 2013). The ammonia produced by rhizosphere bacteria is involved in the supply of nitrogen to plants and phytopathogens through the reduction of plants colonization by pathogens.

2.9.2 Hydrogen Cyanide (HCN) and Siderophore Synthesis

Hydrogen cyanide (HCN) is a secondary metabolite produced commonly by rhizosphere microorganisms. Microbial production of HCN has been reported as an important antifungal

component to control plant infecting fungi directly and plant growth promoting agents indirectly. The production of HCN is common amongst *Pseudomonas* (88.89%) and *Bacillus* (50%) species in the rhizosphere of plants and root nodules of legumes (Ahmad *et al*., 2008). Fluorescent Pseudomonas strain RRS, isolated from a flowering plant produced HCN and the strain improved seed germination and root length (Saxena *et al*., 1996). Ten fluorescent pseudomonads isolated from rhizosphere of sunflower, potato, maize and groundnut were shown to enhance plant growth and suppress the collar rot of sunflower where in one of the mechanism of suppression of the disease was by HCN production (Shivani *et al*., 2005).

Under iron limiting conditions, PGPR produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion. Microbes release siderophores to scavenge iron by forming soluble ferric iron (Fe^{3+}). They are important for none pathogenic bacteria for their iron acquisition (Miethke and Marahiel, 2007). Siderophores play a role in plant disease suppression, which inhibit the growth and metabolic activity of plant pathogens through iron sequestration. Tailor and Joshi (2012) screened siderophore producing bacteria from the rhizosphere and reported that more than 85% of them produce siderophore under *in vitro*, of which *P. fluorescens* was the most efficient producer of siderophore. Rakh et al. (2011) reported that *Pseudomonas* species is an efficient siderophore producer with strong antagonistic activity against *S. rolfsii*. Other soil bacteria such as *Azotobacter* and *Bacillus* are efficient siderophore producers and used to enhance plant productivity through protection from phytopathogens. Siderophores directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of iron to the bacteria that could suppress the growth of pathogenic fungi *viz. F. oxysporum* and *R. solani*, and induce host resistance (Haa and Defago, 2005).

2.9.3 Phosphate Solubilization and Lytic Enzymes Secretion

Microorganisms offer a biological role capable of solubilizing the insoluble inorganic soil phosphorus (P) and make it available to plants. The ability of some microorganisms to convert insoluble P to its accessible form is an important trait of PGPR to increase plant growth and yield (Rodriguez *et al*., 2006). Thus, the phosphate solubilizing bacteria (PSB) could be utilized as a promising source of plant growth-promoting agents. The use of PSB as inoculants increases

phosphorus uptake by plants. The species of *Pseudomonas* and *Bacillus* have been reported as a good PSB in agricultural soils.

Bacteria could control plant pathogenic fungi through secretions of lytic enzymes that attack the cell wall and oospores of the pathogens. Some of the lytic enzymes produced by PGPR are glucanase, proteases, cellulase and chitinase. Additionally, lytic enzymes affect spore germination and germ tube elongation of phytopathogens. A positive relationship was observed between chitinase production and the antifungal activity of chitinolytic *P. fluorescens* isolates (El-Tarabily, 2006).

2.10 Synergy of Rhizospheric Soil Microbes as Inoculants

The co-inoculation of microorganisms is becoming an important component in the management of plant disease in the modern agricultural activity. For instance, the biocontrol potential of *T. harzianum* and *B. subtilis* controlled the incidence of fusarium root rot and increased the growth parameters per plant viz., shoot length, fresh and dry shoot weights, branch number, root length and fresh and dry root weights as well as yield related parameters during field application (Abd-El-Khair *et al*., 2018). The co-inoculation of *T. viride* and *P. fluorescens* inhibited mycelial growth of *P. infestans* and reduced the disease infection of late blight of potato by 36.7% in the greenhouse study (Ephrem Debebe *et al*., 2011). On the other hand, the dual inoculation of seeds with a mixture of *Rhizobium leguminosarum* and *T. viride* tag4 then foliar spraying of the developed plants with the spore suspension of *T. viride* tag4 on the 35^{th} and 55^{th} day from sowing reduced chocolate spot disease and enhanced nodulation, nitrogenase activity and nitrogen fixing bacterial population in the rhizosphere of faba beans (Saber *et al*., 2009).

Trichoderma spp. and *B. subtilis* together showed the highest ability to suppress rice sheath blight caused by *R. solani* and enhanced the productivity of the crop (Ali and Nadarajah, 2013). *Bacillus pumilus* INR7 and *R. intraradices* decreased the disease severity of rhizoctonia root rot of common bean, their blend not only decreased the disease status, but also improved the dry weight and health of the crop (Hussein *et al*., 2018). Field application of *T. harzianum* and *B. subtilis* combined with *Rhizobium* sp. reduced the disease incidence of *F. solani* as pre- emergence and post- emergence damping off as well as root rot of faba bean (Abd-El-Khair *et al*., 2018). The same treatment

increased growth and yield related parameters such as shoot length, both fresh and dry shoot weight, branches number, root length and both fresh and dry root weight, pod number and fresh and dry weight of pod per plant. Under field conditions, the co-inoculation of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 showed 62 and 34% increment of seed dry weight and grain yield over the uninoculated control, respectively in Ashebeka variety. The same treatment showed 56 and 38%, increase of seed dry weight and grain yield, respectively in trial Hachalu variety (Zewdineh Firdu *et al*., 2020).

2.11 Greenhouse and Field Success of Bioinoculants

Bioinoculants are the beneficial soil amendments that use microbes for promoting plant growth and development (Chaudhary *et al*., 2020). Several plant growth-promoting microbial strains such as *Azospirillum, Rhizobium, Bacillus, Pseudomonas* and *Trichoderma* have been identified that are used as bioinoculants (Tahir *et al*., 2017). Plants take benefit from microbes in various ways (1) by PGPR that act as bioinoculants (2) by phytostimulation (phytohormones expressed by microbes like *Azospirillum*) that directly promote the growth of plants (3) by acting as biological control agents (like *Bacillus, Trichoderma* and *Pseudomonas*) that protect plants against harmful organisms (Tang *et al*., 2020). Schematic descriptive mechanism of several traits shown by PGPR is presented below (Figure 1.3).

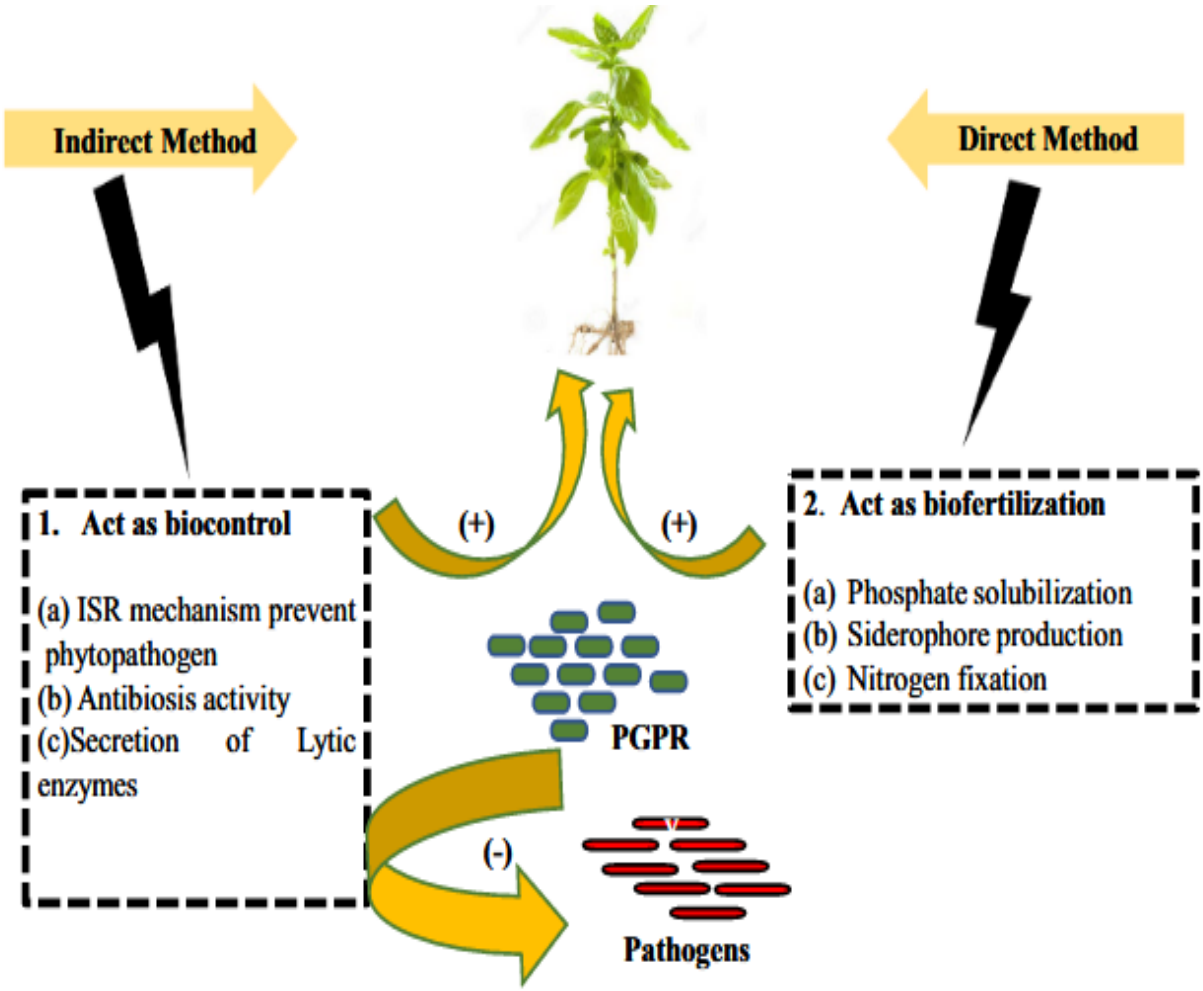


Figure 1.3 Schematic descriptive mechanism of several traits shown by PGPR that acts as bioinoculants. Various mechanisms can be studied by (1) biocontrol of various pathogens (2) acting as biofertilization. As a biocontrol process: (a) Induction of Systemic Resistance (ISR) mechanism by PGPR, (b) Antibiosis activity and (c) Secretion of lytic enzymes. Biofertilization involves (a) Phosphate solubilization, (b) Siderophore production and (c) Nitrogen Fixation (Source: Chaudhary *et al*., 2020).

Bioinoculants contain dormant or live cells of the efficient strain of nitrogen-fixing, hydrogen cyanide and siderophore producing microorganisms. Thus, bioinoculants are being used under greenhouse and field conditions as an inputs of different crops alternatively to biofertilizers and biofungicides. Studies indicated that a combined application of different root-colonizing bio-control agents, such as *Trichoderma* and *Fusarium*, better than a single application of any one

agent. Camacho et al. (2001) have reported an increased nodulation on common bean when *Bacillus* sp. strain CECT 450 was combined with *R. tropici* strain CIAT 899 under controlled and field conditions. They also observed that a single inoculation of CECT 450 produced no significant effect on bean plants. Rokhzadi et al. (2008) have reported that a combined inoculation of *Azospirillum* spp., *Azotobacter chrococcum* 5, *Mezorhizobium ciceri* SWR17, and *Pseudomonas fluorescens* P21 strains improved biomass and grain yields of chickpea under field conditions better than single or combined inoculation without *P. fluorescens* P21. The authors attributed the increase in yield to the cumulative effects of the rhizobacteria, particularly on the supply of nutrients to the crop and the production of growth promoting substances of which *P. fluorescens* P21 played a key role.

2.12 Characteristics of Bioinoculants

In their feature bioinoculants can exhibit the biofertilizers, phytostimulators and biocontrol characteristics. According to Singh et al. (2016) the biofertilizer properties of bioinoculants includes biological nitrogen fixation, phosphate solubilization and phytase production, and siderophore synthesis, and the phytostimulators characters are phytohormones such as the synthesis of auxins, cytokinins, gibberellins, ACC Deaminase and ethylene. The biocontrol properties are antibiosis, hydrogen cyanide, competition for nutrients and niches, lytic enzymes and induction of systemic resistance in plants.

2.13 Formulation of Bioinoculants

The bioinoculant formulation is the uniform mixture of selected beneficial strain with a suitable carrier material that can provide stabilization and protection of strain during transport and storage. The carrier is the vehicle of living latent microbes that provides protection and supportive niche to the microbial community (Chaudhary *et al*., 2020). It is a multistep process that results in one or more strains of microorganisms contained in a particular carrier material together with sticking agents or other additives which help in the protection of the cells during storage and transport time. The formulation process improves the efficiency and shelf life of biofertilizers. Sometimes, the formulation contains some cell protectants with desired microorganisms that increase the shelf life of spores during adverse conditions (Bhattacharyya *et al*., 2020). Different types of formulation

are existing on the basis of their efficiency of which solid and liquid are some of the formulation types.

2.13.1 Solid Formulation

In this preparation, the beneficial bacterial strain is mixed with a solid carrier. It is used for transporting strain from the laboratory to fields. Mainly peat, powder and granules are used for this formulation. It provides a protective and nutritive environment to those microbes that form micro-colonies. Peat should be adaptive, easily sterilized, nontoxic with high water holding capacity (Ceglie *et al*., 2015). After the process of peat drying, it is passed through 250 µm sieves and mix with proper strain. For bacterial multiplication, peat is incubated at a specific temperature and this step is called curing. The main drawbacks of using peat is its variability in its composition and quality. Sometimes toxic compounds are also released from peat during the process of sterilization that can affect the survival rate and growth of microbes (Malusa *et al*., 2012). Recently, granules have been used instead of peat since granules have some advantages over peat. The *Trichoderma* species can be formulated using Talc based, vermiculite-wheat bran, alginate prills, banana waste and coffee husk based formulation (Kumar *et al*., 2014).

2.13.2 Liquid Formulation

The liquid formulation is the microbial preparation that contains those beneficial microbes, which have the capability of solubilizing, fixing or mobilizing essential plant nutrients by biological activities (Bahadur *et al*., 2016). phosphate mobilizing microbes such as *Bacillus* and *Pseudomonas* spp., nitrogen-fixing microbes and many other groups of microbes are used in the liquid formulation (Surendra and Baby, 2016). The liquid formulation is the latest and promising technology over conventional carrier-based formulation due to its beneficial effects. The carrier-based formulation has a short shelf life, i.e., 2-3 months, and it cannot retain throughout the crop cycle (Chaudhary *et al*., 2020). Deep tank fermentation system is employed in liquid formulation of Trichoderma species which make it a more preferred approach for biomass production using inexpensive growth media such as molasses and brewer's yeast (Papavizas *et al*., 1984)

2.14 Methods of Inoculant Applications

The success of microbial inoculation depends largely on the following: the plant species and cultivar, soil type, soil moisture and temperature conditions, the number of pathogens present in the soil around the plant and how the inoculants were prepared and applied (Babalola *et al*., 2007). The application methods are given below (Table 1.5).

Table 1.5. Methods of Bioinoculant Applications

Methods	**Mechanisms of application**	**Advantages and limitation**	**References**
Directly to the soil	After seed germination, they are applied directly to the soil at the plant base near the plant roots	Withstand low moisture conditions better than carrier based inoculants. A less expensive method	Mokone and Babalola (2013)
Seed application	Seeds are coated with microbe-carrier slurry. Adhesive solution such as sucrose solution is recommended.	Adequate loading of bacterial cells. Microbial culture may move away from rooting zones after application	Akladious and Abbas (2012)
Seedling root dipping	The roots of the seedling are dipped in a mixture of microbial culture and water for 5-10 min and transplanted	A less expensive method compare to carrier base inoculants	Babalola et al.(2007)
Field/Soil application	Generally the granular inoculants are placed on the furrow under or alongside the seed.	less time consuming than the seed inoculation method	Babalola et al.(2007)
Broadcasting	Microbial inoculants could also be mixed with farmyard manure before broadcast	Rapid and greater colonization of inoculants per unit area	Akladious and Abbas (2012)

CHAPTER 2

Isolation and Characterization of antagonistic bacteria from the rhizosphere of faba bean (*Vicia faba* L.) for their antagonistic potential against *Botrytis fabae* and to assess their multiple plant growth-promoting properties of the host under *in vitro* conditions

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Abstract

Chocolate spot is one of the major fungal diseases of faba beans caused by *Botrytis fabae* and cause 67.5% yield loss in Ethiopia. The use of eco-friendly approach is an alternative to fungicides that involves the application of phytobeneficial bacteria. Thus, this study was conducted to evaluate the antagonistic potential faba bean rhizosphere bacterial isolates on *B. fabae* and assess their plant growth-promoting traits under *in vitro* conditions. The data were analyzed by SPSS version 24. In dual culture assay, different bacterial isolates inhibited *B. fabae* (6-68%) upon 3-9 days. The isolates showed 6-82% of *B. fabae* inhibition using culture filterate (5-20%). Isolate AAUB95 and AAUB100 (80%) were the best antagonistics with 82% and 100% of *B. fabae* inhibition, respectively at 20% of the filtrate. In addition, 75, 60,65 and 60% of the isolates were positive for protease, lipase, indole-3-acetic acid and ammonia, respectively. The quantitative assay of tricalcium phosphate (TCP) solubilization produced 200-760 µg mL^{-1} upon 3-6 days. The change in the pH of National Botanical Research Institute's phosphate growth medium (NBRIP) was related to TCP solubilization and the drop in pH value was seen from the 3^{rd}-6^{th} days revealing an inverse relationship (r= -0.422*) to each other. Based on 16S rRNA sequence analysis, isolate AAUB95, AAUB146b and AAUB100 were identified as *Bacillus subtilis* AAUB95, *Serratia nematodiphila* AAUB146b, *and B. tequilensis* AAUB100, respectively. Of these strains *B. subtilis* AAUB95 showed the best antagonistic feature on *B. fabae* with plant growth-promoting traits production.

Keywords: *Bacillus* spp.; *Botrytis fabae*; Dual assay; phosphate solubilization; protease

2.1 Introduction

Chocolate spot (*Botrytis fabae*/*cinerea*) is one of the major fungal diseases affecting faba bean production in central Ethiopia. It is widely expanding to different parts of the country compared with other fungal diseases such as ascochyta blight, faba bean rust and root rot (Dagne Kora *et al*., 2017). Different reports showed that yield loss caused by the epidemics of chocolate spot can reach as high as 50-100% on highly susceptible faba bean varieties in Australia, Egypt, England and China (Saber *et al*., 2011; Abo-Hegazy *et al*., 2012). In Ethiopia, the yield losses of up to 67.5 % was recorded in susceptible faba bean cultivars by chocolate spot disease (Samuel Sahile *et al*., 2010).

Farmers use fungicides such as MORE 720 WP (Mancozeb+Cymoxanil), and ORZEB 80WP (Mancozeb) to control chocolate spot and other foliar fungal diseases of faba bean (Kabbabeh *et al*., 2009). However, the continuous application of these fungicides enhanced the resistance of the pathogen and residual chemical in faba bean seeds which is implicated with health hazards (Hassan *et al*., 2006). This necessitates the importance of integrated pest management that combine good agricultural practices such as changing irrigation and crop rotation to reduce pest problems, chemical application and along with the use of biological control agents.

Biological control (BC) is a tool of pest and disease management of different crops that involves the application of selected soil microorganisms such as bacterial and fungal biopesticides (Deshmukh *et al*., 2010). Soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues and stimulate plant growth are collectively known as plant growth promoting rhizobacteria (Vessey, 2003). These useful microorganisms are used with the aim of improving crop yields by augmenting nutrient availability, enhancing plant growth and protection of plants from diseases and pests. The species of *Bacillus*, *Pseudomonas*, *Serratia*, *Enterobacter*, *Burkholderia*, *Agrobacterium*, *Streptomyces*, etc. are the well-studied bioprotectants and plant growth-promoting rhizobacteria.

These plant growth-promoting rhizobacteria (PGPR) protect plants from pathogens through the production of lytic enzymes such as protease, lipase, cellulase, chitinases and induction of systemic resistance. Besides, PGPR promote plant growth directly by synthesis of phytohormones such as,

indole-3- acetic acid (IAA) or indirectly via modifying microbial balance in the rhizosphere, favoring the beneficial ones and phosphate solubilization (Gupta *et al*., 2015). They persist in the soil, particularly on the rhizosphere, eventually or as endophyte guarantee their long-term advantages provided to plants (Woo *et al*., 2014).

There are numerous findings that indicated the antagonistic potential of rhizobacterial isolates against *Botrytis fabae*. Most of the effective antagonistic studies were obtained from *Bacillus* and *Pseudomonas* spp. Consequently, different researchers showed that bacterial isolates; *Bacillus macerans* (Sharga, 1997), and combined inoculation of *P. fluorescens* and *B. subtilis* (El-Banoby et al. (2013) reduced the disease incidence of chocolate spot by 33% and 58%, respectively. Ahmed (2015) and El-Banoby et al. (2013) have reported the inhibitory activity of *B. subtilis* against the mycelial growth of *B. fabae by* 62.6% and 67.03%, respectively. In Ethiopia, Fekadu Alemu (2016) has showed that *P. fluorescens* isolates with 88% mycelial growth inhibition of *B. fabae* in *vitro;* whereas Samuel Sahile et al. (2009) has showed that isolates of *Bacillus* spp. reduced the growth of *B. fabae* by 23-64% under *in vitro* conditions.

Arsi and Bale zones are also parts of the major faba growing areas in Oromia region, Ethiopia and are some of the hot spots faba bean chocolate spot disease (Dagne Kora *et al*., 2017). At present time, the symptom of chocolate spot is being widely seen on faba bean crops in Eastern Arsi at Digeluna Tijo, Tiyo districts and in Bale zone of Goba district, indicating the prevalence of the disease in the districts.

Therefore, the present study was initiated to evaluate the *in vitro* inhibitory potential of rhizosphere bacterial isolates against *B. fabae*; to assess the mechanisms used to antagonize the study pathogen and additionally to determine the plant growth-promoting characteristics exhibited by the isolates.

2.2 MATERIALS and METHODS

2.2.1 Description of the Study Areas

Soil samples were collected from Arsi zone (at DigelunaTijo and Tiyo districts) and Bale zone (Goba district). DigelunaTijo is located at geographical coordinates of 7° 19' 60.00" N latitude and 39° 14' 60.00" E longitude and altitude between 2500-3560 meter above sea level (a.s.l). The district is found in Arsi zone, bordered by Bekoji on the South, Munesa on the South-West, Tiyo on the North-West, Hitosa on the North, Tena on the North-East and Sherka on the East. It has 39.5% arable, 27.4% pasture, 13.3% forest and 19.8% as swampy lands (SEP, 2019). Tiyo district is found at 7° 49' 59.99" N latitude and 39° 09' 60.00" E longitudes, altitude of 1780-3100 m.a.s.l. The district is bordered by Munesa on the South, Ziway Dugda on the West, Hitosa on the North-East and Digeluna Tijo district in the South-Eastern part. This district has 40% arable, 23.1% pasture, 8.7% forest and 28.2% as a swampy land (SEP, 2019) and altitude of 1780-3100 m. a. s. l, with a temperature of 5-28°C and relative humidity of 43-60%. Both DigelunaTijo and Tiyo districts have bimodal rainfall having March to April short rainy and July to October, long rainy seasons.

Goba district is found in Oromia region, Bale zone. It is bordered on the South by Mennana Harena Buluk, on the West by West Arsi, on the North by Mena River and on the South-East by Berbere town with coordinates of 6° 49' 59.99" N latitude and 39° 49' 59.99" E longitude. This district is located at a distance of 14km South of Bale zonal town, Robe at 444km South-East of Addis Ababa, with coordinates of 6° 49' 59.99" N latitude and 39° 49' 59.99" E longitude. The altitudes of this district is 1500 - 4377 m.a.s.l, having a temperature of 0-23°C (BZMED, 2007). As a part of Bale zone, Goba District has two types of rainfall regime. The long rainy season extends from March to April with high rain fall during June, July and August. The second rainy season of rain fall regime is influenced by equatorial westerly and easterly winds with rainfall during spring and autumn. The major soil types are Chromic and Pellic Vertisols in some parts, Chromic, Orthic and Vertic Luvisols around highlands and plateaus areas. A survey of the land in this District shows that 13% is arable or cultivable, 27.6% pasture, 54.6% forest (or part of the Bale Mountains

National Park), and the remaining 4.8% is considered degraded or otherwise unusable (BOARD, 2012).

For this study, Arsi and Bale zonal area were selected because the symptom of chocolate spot was prevalently observed on faba bean crops under field conditions, indicating the presence of *Botrytis* spp. (*B. fabae* and *B. cinerea*) in the areas (Figure 2.1). These sampling districts were selected because the standing faba bean crops were severely infected by chocolate spot disease (Dagne Kora *et al*., 2017; Shiferaw Deneke *et al*., 2018).

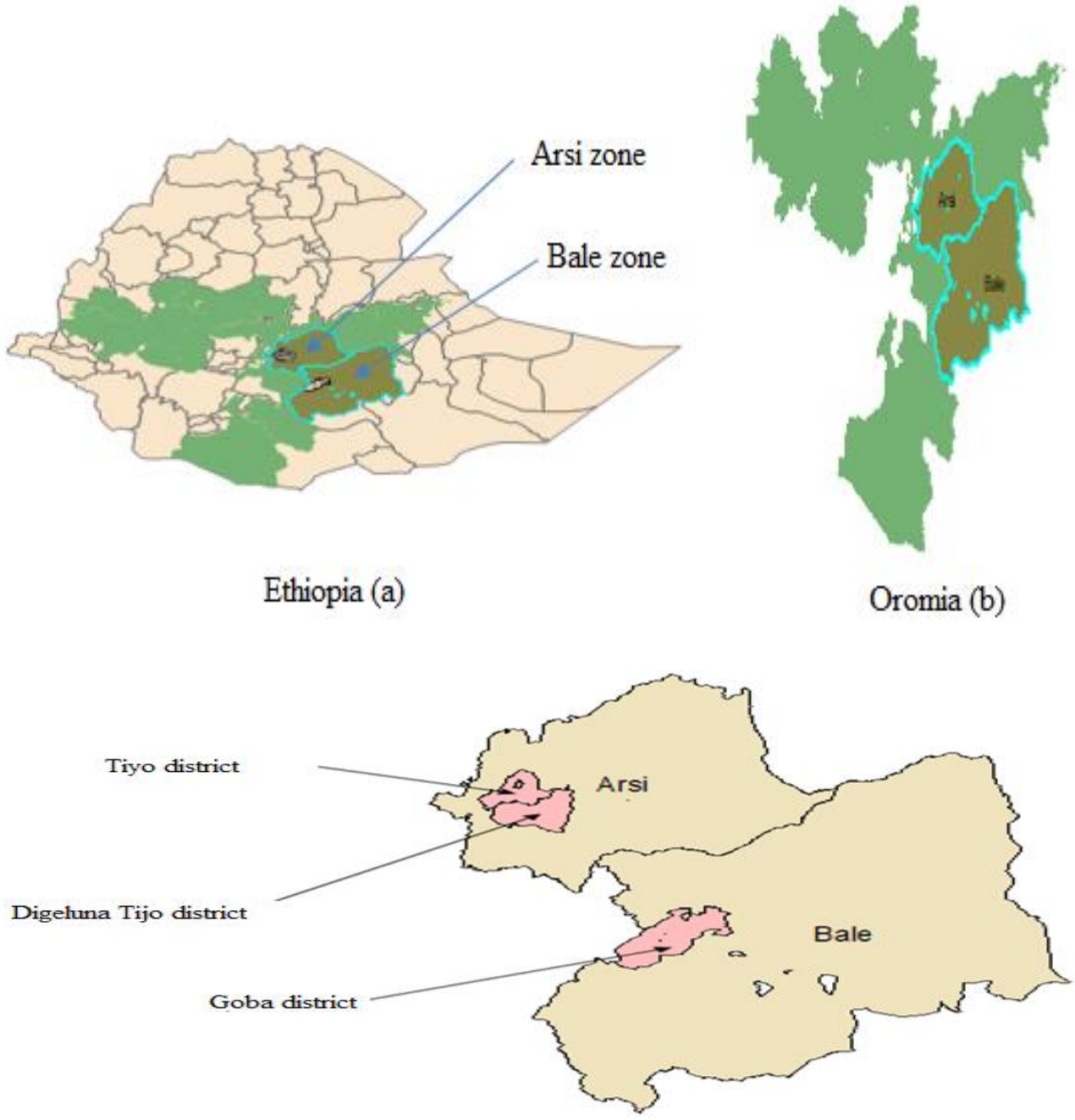

Figure 2.1: A map showing the study zones, districts and sampling areas of the study districts

2.2.2 Soil Sampling and Processing

Rhizospheric soil samples were collected from the study areas during faba bean growing season of 2016. The samples were coded as FRSS-01, FRSS-02, FRSS-03, FRSS-04, FRSS-05, FRSS-06, FRSS-07, FRSS-08, FRSS-09, FRSS-10, FRSS-11, FRSS-12, FRSS-13, FRSS-14, FRSS-15 and FRSS-16. "FRSS"- stands for faba bean rhizosphere soil samples with corresponding number. From each sampling site, faba bean plants were uprooted and about 50 g of root adhering soil samples were collected from the standing healthy faba bean plants, in triplicates, and composited in one and collected in alcohol-surface sterilized polyethylene bags and stored at room temperature for the isolation of rhizosphere bacterial species.

2.2.3 Isolation of Rhizobacterial Isolates

Rhizobacteria were isolated from collected soil samples as described by Walker et al. (1998). Ten gram (10 g) of soil sample from each site was suspended in 90 mL of 0.85% saline water in 250 mL flasks, and shaken on orbital shaker at 120 rpm for 30 min. The suspensions were further diluted to 10^4-10^7. From appropriate dilution factor, 100 µL was transferred to nutrient agar medium modified with 1% dextrose, and 100 µg Amphotericin. They were prepared in triplicates, and incubated at 28$\pm$2°C for 24-72 hrs. The number of colonies per plate was counted and, representative colonies were picked and sub-cultured to obtain pure isolates. The purified isolates were preserved in 20% glycerol and incubated at -20°C for further studies.

2.2.4 Isolation of the test pathogen (*Botrytis fabae*)

The test pathogen was isolated from leaves (showing symptoms of chocolate spot) of faba bean collected from the study areas and seeds from storage according to Shinde (2016) using faba bean dextrose agar (FDA) medium (200 g faba bean seed extracts, 20 g dextrose, and 18 g agar). Seeds and leaves were washed under tap water, surface sterilized with 3% sodium hypochlorite for 5 min and rinsed in sterilized-distilled water and air dried. Four to six seeds and leaf cuts per plate were transferred to FDA and incubated at room temperature for 10-12 days under 12 hrs. light and 12

hrs. dark conditions and purification was done by sub-culturing on potato dextrose agar (PDA) plates. The identification of *B. fabae* was conducted through looking at the cultural characteristics (i.e. colony color, spores, conidia structures and formation of sclerotia on the PDA). The purified culture was then preserved at 4°C using PDA slants and utilized for the screening of pre-isolated bacterial species.

2.2.5 Pathogenecity test of the test Pathogen (*B. fabae*)

The pathogenecity test was done according to Abdel-Aleem et al. (2011). Faba bean seeds were surface sterilized with 3% sodium hypochlorite for 5 min and rinsed in sterilized-distilled water. Faba bean seeds were sown in 3 kg alchol sterilized pots filled with sterile soil. Fifteen days after planting, 12 mL of the spore suspension was adjusted to $4.5x10^5$ spores/mL using haemocytometer, sprayed on the foliar parts of faba bean seedlings and covered with surface sterilized plastics, whereas the control was sprayed with 12 mL sterilized water. The occurrence of chocolate spot diseases infection on faba bean seedlings was checked interms of its intensity and severity visually and the infected plants parts were cut and used to re-isolate the pathogen after 7^{th} days of spraying to confirm the Koch's postulate. The isolates were tested for cultural characteristics and compared with the reference strain of *B. fabae* obtained from the Phytopathology Laboratory of the Ethiopian Institute of Agricultural Research (EIAR).

2.2.6 Cultural characterization of Bacterial Isolates

The bacterial isolates were gram stained by taking a loopful of 24 hrs. culture on glass slides and heat fixed. The slides were flooded with crystal violet, for 1 min, rinsed with water. They were mordant dyed with iodine for 1 min and counter stained with Safranin after washing with water and observed under microscope (OLYMPUS-BX51, Germany) of oil immersion objective to distinguish the gram reaction of the bacteria cells.

2.3 Molecular Identification of the Bacterial Isolates

2.3.1 Extraction of DNA

The extraction of bacterial DNA was conducted according to Souza et al. (2017) for all isolates that showed antagonism to *B. fabae* and plant growth promoting properties. In this method for the genomic DNA extraction, preserved strains were cultivated on nutrient agar medium at 25°C for 48 hrs. The cultures were transferred to sterilized 1.5 mL micro centrifuge tubes containing 200 µL of cell lysis buffer (0.05 M NaOH; 0.25 % SDS). The micro tubes were heated at 100°C for 20 min. The micro centrifuge tubes containing the samples were centrifuged at 10,000 rpm for 3 min and the sediment was diluted 20x in sterile MilliQ water and stored at -20°C for PCR reactions.

Since the identification was not definitive yet, the isolates were tentatively identified by taking the highest similarity percentage into consideration and presented with "AAUB" and the corresponding "isolation number" for this study. The 16S rRNA gene, ~1.5 kilo base (kb) in length, has proven to be a useful molecular target since it is present in all bacteria, either as a single copy or in multiple copies, and it is highly conserved over time within a species (Sabat *et al*., 2017). All the identified isolates are preserved at Addis Ababa University in the applied microbiology laboratory and University of Lavras, culture collection center, Brazil.

2.3.2 Quantity and Quality Determination of DNA

The quantity of the extracted genomic DNA was determined by measuring the absorbance at 260 nm using Thermo Scientific Nano Drop (Nano Drop 2000). The quality and suitability for PCR reaction of the DNA was checked by visual comparison with the standard DNA size markers after electrophoresis through 0.8% agarose TAE (Tris-acetate EDTA) gels and stained with 0.5 mg mL^{-1} ethidium bromide (Sigma Chemicals Co.).

2.4 Primers and PCR Conditions for Bacterial DNA Amplification (16S rRNA)

For PCR reactions, 6 µL of the extracted DNA was utilized as a template for the amplification 16S RNA. The amplification of the 16S rRNA gene was performed using a T1-Thermocycler PCR

machine with 8NF (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1429R (5`-ACGGCTACCDTTGTTACGACTT-3`) (Esikova *et al*., 2002). The total volume of PCR reaction used was 25 µL. PCR amplifications were performed with 10 µL (Gotaq polymerase), 0.56 µL forward primer, 0.56 µL reverse primer, 0.40µL (bovine serine albumin, 7.48 µL of nuclease-free water and 6 µL DNA template (Table 2.1). The condition of PCR complied with the method of Souza et al. (2017). The PCR amplification cycle included an initial 2 min denaturation at 95°C followed by 10 cycles of denaturation at 95°C for 1 min, 1 min primer annealing at 65°C with the annealing temperature decreased by 1°C with each succeeding cycle, and 1 min elongation at 72°C. The denaturation phase, annealing and elongation were conducted for 35 cycles. The PCR products were visualized on ethidium bromide staining at 1.5% agarose gel electrophoresis and the amplicons were purified using GeneiPure^{TM} quick PCR purification kit (GeNei^{TM}, Bengaluru, India) spectrophotometer taking calf thymus DNA as control. The purified partial 16S rRNA amplicons sequenced was performed at Macrogen Inc. (Seoul, Korea). The sequence data were assembled and analyzed using the Lasergene sequence analysis software package (DNA Star, Inc. Madison, WI, USA).

Table 2.1: Components and amounts of PCR mixtures used for DNA amplifications

S.N	Components	Amount (µL)
1	Gotaq polymerase	10.00
2	Primer 1(8NF)	0.56
3	Primer 2 (1429R)	0.56
4	Bovine serine albumin (BSA)	0.40
5	ddH_2O	7.48
6	DNA template	6.00
Total volume of reaction		**25.00**

2.4.1 Computational Analysis (BLAST) and Phylogenetic Tree Construction

The sequences obtained were blasted against the GenBank database using the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov). The similarity of the blasted

sequences was identified from the best score of the compared species from National Center of Biotechnology Information (NCBI). The nucleotide sequences were aligned using multiple alignment fast Fourier transform, MAFFT (Katoh *et al*., 2002). The phylogenetic tree of bacterial isolates was constructed by molecular evolutionary genetic analysis (MEGA) version X. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to construct the phylogenetic tree using K2+G+I model.

2.5 Screening for Antagonism against the test Pathogen (*B. fabae*)

2.5.1 Dual Culture Assay

The dual culture screening of bacterial isolates was done according to Zivkovic et al. (2010). A loopful of bacteria from 48 hrs. was streaked on the opposite side of the petri plate containing a PDA modified with 10% sucrose and followed by incubation at 28±2ºC. After 48 hrs. of incubation, 5 mm mycelial disc from 9-days old *B. fabae* was inoculated on the opposite side of bacteria on the same plate and were incubated until the control plates were fully covered by the growth of *B. fabae*. The measurement of radial growth was taken on 3^{rd}, 5^{th}, 7^{th} and 9^{th}days of incubation. The assay was performed in triplicates and the percent inhibition of radial growth (PIRG) was calculated by using the following formula (Zivkovic *et al*.,2010).

$PIRG = R_1\text{-}R_2/R_1X\ 100\%$

Where, PIRG-percent inhibition of radial growth, R_1- the radius of *B. fabae* in the control and R_2- radial growth of *B. fabae* toward the bacterial spp. in the treated Petri plates.

2.5.2 Culture Filtrate-None Volatile Assay

The bacterial isolates that inhibited the growth of *B. fabae* under dual culture assay were further screened using culture free supernatant according to Haggag and El Soud (2012). The bacterial isolates were grown in nutrient broth at 28±2^{o}C on a rotary shaker (ZJZD-III, Shanghai, China) at 150 rpm for 48 hrs. The cultures were centrifuged (Centrifuge, Wagtech international, United

Kingdom) at 4000 rpm for 30 min. The supernatants were sterilized by passing through sterile filter papers (0.2 mm), and each culture filterate was added in a sterilized PDA medium amended with 10% sucrose at concentrations of 5, 10,15 and 20% (v/v). The plates were then centrally inoculated with 5 mm mycelial disc of *B. fabae* from 7 days old culture and incubated at 25±2^{o}C. Three replicates were made for each treatment and the control plate without pathogen. The percent inhibition of mycelial growth (PIMG) was calculated according to Begum et al. (2008) using the following formula.

$PIMG = D_1\text{-}D_2/D_1X100$

Where, D_1-the diameter of *B. fabae* in the control and D_2- the diameter of *B. fabae* in the treated plate

2.5.3 Hydrogen Cyanide Assay

Hydrogen cyanide (HCN) production was detected according to Bakker and Schippers (1987). Bacterial isolates were grown on HCN induction medium (5 g/L Tryptic Soy broth, 0.88 g/L glycine and 3 g/L Agar**)** for 2-5 days at 28±2°C. A 100 µL ($1x10^6 CFUmL^{-1}$) was spread at the center of the petri plate. A sterilized filter paper (Whatman No. 1) strips was impregnated in picric acid solution (2.5 g of picric acid and 12.5 g of Na_2CO_3) and placed on the lid of the plates. Plates having the filter paper only was considered as controls. Petri plates were sealed with parafilm and incubated at 28±2°C for 48-72 hrs. A change in colour of the filter paper strip from yellow to orange-brown was considered positive for HCN production.

2.5.4 Ammonia Production Assay

The qualitative assay of ammonia production by microbes was done according to Cappuccino and Sherman (1992). The bacterial isolates were grown in 5mL peptone water at 28$\pm$2°C for 4 days. Following the incubation, 1 mL of Nessler's reagent ($HgCl_2$, 10.0 g, KI, 7.0 g, NaOH, 16.0 g, H_2O (ammonia free), 100 mL) was added to the cultures to detect the development of yellow color which indicates the production of ammonia.

2.6 Other plant growth-promoting characteristics of the Rhizobacteria

2.6.1 Qualitative and quantitative Tricalcium phosphate (TCP) solubilization

Qualitative study of phosphate solubilization was conducted using Pikovskaya's (PVK) agar medium and quantitative assay was determined according to Nautiyal (1999) using National Botanical Research Institute Phosphate (NBRIP) medium containing tricalcium phosphate as the sole source of phosphate. The medium contained; (g/L), glucose (10.0), $Ca_3(PO_4)_2$ (5.0), $MgCl_2.6H_2O$ (5.0), $MgSO_4.7H_2O$ (0.25), KCl (0.2) and $(NH)_2SO_4$ (0.1). Phosphate solubilization index (PSI) was calculated according to Silva Filho and Vidor (2000).

$$PSI = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

A 500 µL of the bacterial cultures ($1x10^8$CFU mL^{-1}) was inoculated into 50 mL NBRIP broth and incubated on orbital shaker (ZJZD-III, Shanghai, China) with 150 rpm at room temperature. Uninoculated sterilized NBRIP was included as control and incubated for 9 days (Chagas *et al*., 2015). Contents of the culture were withdrawn, centrifuged (Centrifuge, Wagtech international, United Kingdom) at 10,000 rpm for 25 min on the 3, 6 and 9 days of incubation to measure the free phosphorus released and pH from 1 mL of the supernatant. The amount of released phosphorus was detected using spectrophotometer (6405UV/Vis., Jenway, England) at 880 nm and the concentration of released phosphate was quantified against a standard curve constructed from known quantity of potassium di-hydrogen orthophosphate, KH_2PO_4, (µg mL^{-1}). The pH of the supernatant was also measured on the same time interval with a digital pH meter (NIG 333, New Delhi, India).

2.6.2 Qualitative and Quantitative Assay of Indole-3-Acetic Acid (IAA)

The ability of isolates to produce IAA was checked qualitatively as described by Hartmann et al. (1983). A 200 µL bacterial culture ($1x10^8$ CFUmL^{-1}) was grown in 50 mL of in nutrient broth amended with tryptophan (1 g L^{-1}). The culture was, incubated at 25°C on an orbital shaker (ZJZD-III, Shanghai, China) for 72 hrs. Uninoculated tubes served as controls. The culture was

centrifuged (Centrifuge, Wagtech international, United Kingdom) at 3000 rpm for 30 min, from which 2 mL of the supernatants were mixed with (two drops of orthophosphoric acid and 4 mL of Salkowaski`s reagent (50 mL of 70% per chloric acid and 1 mL of 0.5 M $FeCl_3$ and 49 mL sterilized distilled water), and incubated in dark for 30 min. The development of pink colour was visually checked as an indicator of IAA production. One milliliter (1 mL) of the supernatant was quantified spectrophotometrically (6405UV/Vis., Jenway, England) at 530 nm. The concentration of IAA produced was calculated in µg mL^{-1} using the standard curve sketched from known concentrations of indole acetic acid. The standard curve was made using nutrient broth medium amended with tryptophan at 0, 20, 40, 60, 80 and 100 (µg/mL) using acetone as a solvent of standard solutions preparation (Gordon & Weber,1951).

2.7 Enzymatic Assays of the Isolates

Protease activity

The protease activity of the ability of bacterial species was done according to Cattelan et al. (1999) by using skim milk agar medium and incubated at 28±2°C for 3-5 days. The occurrence of clear halo zone around the colony was an indicator of protease activity.

Lipase activity-

The lipase producing potential of the bacterial isolates was performed according to Omidvari (2008). The isolates were grown on nutrient agar medium amended with egg yolk and incubated at 28±2°C. The culture was flooded with saturated aqueous solution of copper sulphate ($CuSO_4$) and kept for 10-15 min. to check for the formation of greenish blue colour zones around the colony and taken as a positive for lipase synthesis.

2.8 Data Analysis

The statistical analysis was performed by One-Way ANOVA of SPSS version 24. The comparisons among means were done by using Student-Newman-Keuls (S-N-K) and Tukey HSD analysis at $\alpha = 0.05$. Values were considered significant at $p<0.05$.

2.9 Results and Discussion

Based on the gram reaction, the rhizobacterial isolates were grouped into Gram positive (45%) and Gram negative (55%) (Table 2.2). The gram negative isolates were grouped into the genera of *Acinetobacter, Enterobacter, Serratia*, and *Enterococcus,* and the gram positive isolates into the genera of *Bacillus* and *Arthrobacter.* The genus *Bacillus* was the dominant rhizobacteria with eight (8) species representing 40% of the isolates, followed by *Serratia* represented with 5 species covering 20% of the isolates. It is interesting to note that the commonest genus *Pseudomonas* contained only one representative isolate.

The 16S rRNA sequence analysis assigned the isolates into the genera of *Bacillus, Serratia, Enterobacter, Arthrobacter, Acinetobacter* and *Enterococcus* (Table 2.2). However, three isolates (AAUB152, AAUB150 and AAUB113b3) was not identified by the sequence analysis, whose DNA amplification failed which might be associated with the primers and/or the PCR conditions employed. However, these isolates were identified to the genus level by treating with a temperature of 70°C for 14 min to check for temperature tolerance and able to grow when re-streaked on nutrient agar to be considered as *Bacillus* genera and inability to survive high temperature and not able to grow either on nutrient agar and King B medium for *Pseudomonas* species.

Table 2.2: Identification and Gram staining of bacterial isolates obtained from faba bean rhizosphere of Arsi and Bale zones

Isolates	The nearest species obtained from GenBank (16S rRNA)	Similarity (%)	Accession number	Gram staining	Identified species
AAUB100	*B. tequilensis* 10b	99.9	HQ223107.1	+	*B. tequilensis* AAUB100
AAUB113b	*S. nematodiphila* DZ0503SBS1	99.7	EU036987.1	-	*S. nematodiphila* AAUB113b
AAUB146b	*S. nematodiphila* DZ0503SBS1	99.7	EU036987.1	-	*S. nematodiphila* AAUB146b
AAUB92	*B. tequilensis* 10b	99.8	AJ276351.1	+	*B. tequilensis* AAUB92
AAUB113c	*S. nematodiphila* DZ0503SBS1	99.7	EU036987.1	-	*S. nematodiphila* AAUB113c
AAUB53	*E. hormaechei* CIP 103441	99.2	AJ508302.1	-	*E. hormaechei* AAUB53
AAUB122a	*B. macroides* LMG 18474	99.2	AJ628749.1	+	*B. macroides* AAUB122a
AAUB122b	*B. subtilis* DSM10	99.9	AJ276351.1	+	*B. subtilis* AAUB122b
AAUB115	*S. nematodiphila* DZ0503SBS1	99.7	EU036987.1	-	*S. nematodiphila* AAUB115
AAUB94	*E. hormaechei* CIP 103441	99.2	AJ508302.1	-	*E. hormaechei* AAUB94
AAUB95	*B. subtilis* DSM10	99.9	AJ276351.1	+	*B. subtilis* AAUB95
AAUB77	*S. nematodiphila*	99.7	EU036987.1	-	*S. nematodiphila* AAUB77
AAUB55	*E. hormaechei* CIP 103441	98.6	AJ508302.1	-	*E. hormaechei* AAUB55
AAUB152	-	-	-	-	*Pseudomonas* sp. AAUB152
AAUB130b	*A. johnsonii* ATCC 17909T	99.2	Z93440.1	-	*A. johnsonii* AAUB130b
AAUB130a	*A. johnsonii* ATCC 17909T	99.2	Z93440.1	-	*A. johnsonii* AAUB130a
AAUB47	*E. faecalis*	99.9	AB012212.1	+	*E. faecalis* AAUB47
AAUB146a	*A. nicotinovorans* X80743.1	99.7	X80743.1	+	*A. nicotinovorans* AAUB146a
AAUB150	-	-	-	+	*Bacillus* sp. AAUB150
AAUB113b3	-	-	-	+	*Bacillus* sp. AAUB113b3

In **"AAUB"**-AAU-indicates the place where isolation was performed representing "Addis Ababa University" and B- imply for "bacteria".

The phylogenetic tree of all the isolate is presented below having some related representative of reference strains retrieved from NCBI (Figure 2.1).

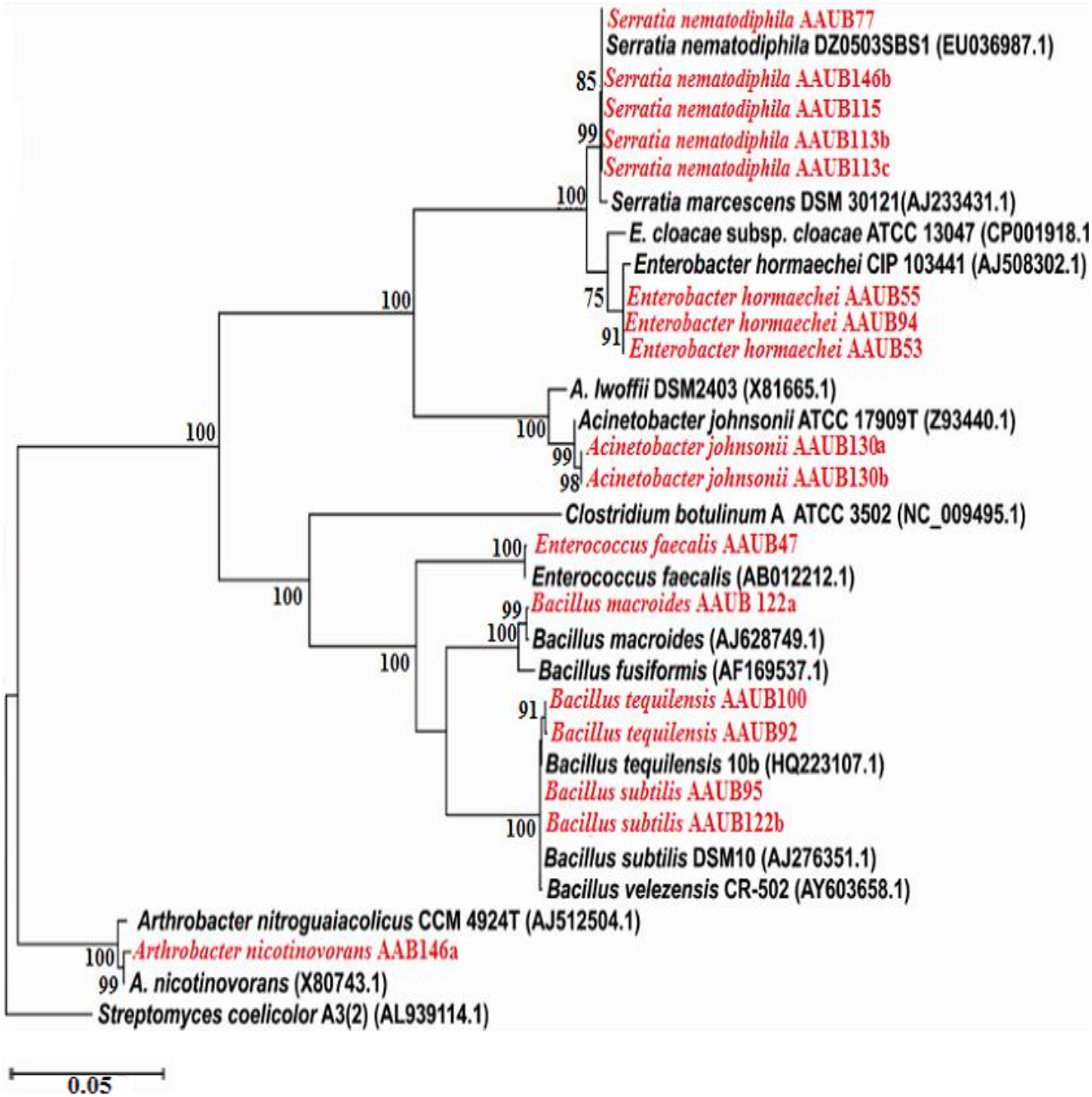

Figure 2.2: Neighbor-joining tree based on the 16S rRNA sequences analysis of the strains and some related representative of reference strains retrieved from NCBI. The numbers on the tree indicate the percentage of bootstrap based on **1000** replications and are shown for branches with more than **75%** support. The **scale bar** represents **0.05** nucleotide substitution per sequence position. Isolates presented in **red color** are obtained from this study. ***Streptomyces coelicolor*** A3(2) (AL939114.1) was used to root the phylogenetic tree.

In the dual culture assay, the rhizobacteria isolates showed a very significant difference ($p<0.05$) in inhibiting the test pathogen with percentage of radial inhibition ranging from 6 to 68% (Table 2.3). Upon 3 days of incubation the isolates inhibited the test pathogen (6-40%) and further increased the inhibition percentage (9-43%) in five days, 16-50% in 7 days, and 24-68% in 9 days of incubation. Rhizobacterial isolates *B. subtilis* AAUB95, *B. macroides* AAUB122a and *B. subtilis* AAUB122b displayed the highest inhibition within short incubation time (3 days) up to the 9 days, showing consistency in their inhibitory activity on the test pathogen. *B. subtilis* AAUB92 displayed the best inhibition within short period (3 days), whereas *S. nematodiphila* AAUB113b showed the highest antagonistic activity starting from 5 days of incubation but *B. tequilensis* AAUB100, *S. nematodiphila* AAUB115, *Bacillus* sp. AAUB150 were characterized with mild inhibition consistent in all the incubation period. Most of the other isolates did not show significant antagonism as a function of time.

Table 2.3: Percent inhibition of radial growth (PIRG) displayed by the antagonistic rhizobacterial isolates against *Botrytis fabae* upon 3-9 days of incubation using dual culture assay

Sample	Isolate code	Species	Percent of inhibition over control in days			
			3 days	5 days	7 days	9 days
FRSS-04	AAUB100	*B. tequilensis*	19^{bc}	25^{cd}	41^{abc}	59^{ab}
FRSS-01	AAUB113b	*S. nematodiphila*	20^{bc}	32^{bc}	52^{a}	57^{abc}
FRSS-08	AAUB146b	*S. nematodiphila*	10^{cd}	17^{de}	30^{bcd}	53^{abc}
FRSS-10	AAUB92	*B. subtilis*	28^{ab}	30^{bc}	40^{abc}	56^{abc}
FRSS-15	AAUB113c	*S. nematodiphila*	12^{cd}	17^{de}	22^{cd}	31^{def}
FRSS-02	AAUB53	*E. hormaechei*	06^{d}	09^{e}	16^{d}	31^{def}
FRSS-13	AAUB122a	*B. macroides*	40^{a}	48^{a}	48^{ab}	68^{a}
FRSS-06	AAUB122b	*B. subtilis*	37^{a}	50^{a}	50^{ab}	55^{abc}
FRSS-03	AAUB115	*S. nematodiphila*	20^{bc}	40^{ab}	40^{abc}	46^{bcd}
FRSS-07	AAUB94	*E. hormaechei*	13^{cd}	23^{cde}	24^{cd}	24^{f}
FRSS-16	AAUB95	*B. subtilis*	30^{ab}	39^{ab}	47^{ab}	68^{a}
FRSS-11	AAUB77	*S. nematodiphila*	08^{cd}	15^{de}	25^{cd}	45^{bcd}
FRSS-14	AAUB55	*E. hormaechei*	12^{cd}	14^{de}	18^{d}	23^{f}
FRSS-08	AAUB152	*Pseudomonas* sp.	13^{cd}	20^{cde}	34^{abcd}	53^{abc}
FRSS-05	AAUB130b	*A. johnsonii*	14^{cd}	16^{de}	21^{cd}	25^{f}
FRSS-07	AAUB130a	*A. johnsonii*	10^{cd}	14^{de}	23^{cd}	41^{cde}
FRSS-09	AAUB47	*E. faecalis*	16^{cd}	19^{cde}	20^{cd}	22^{f}
FRSS-12	AAUB146a	*A. nicotinovorans*	15^{cd}	19^{cde}	20^{cd}	27^{ef}
FRSS-03	AAUB150	*Bacillus* sp.	13^{cd}	25^{cd}	34^{abcd}	50^{bc}
FRSS-11	AAUB113b3	*Bacillus* sp.	10^{cd}	14^{de}	24^{cd}	31^{def}
	CV		0.12	0.10	0.11	0.12

FRSS"- stands for faba bean rhizosphere soil samples with corresponding number and CV-indicates the coefficient of variation among means in the same column. Mean values in the same column labeled with the same letter(s) as superscript are not significantly different ($p>0.05$) by Tukey HSD analysis of One-Way ANOVA.

This assay indicated variability in PIRG potential displayed by different PGPR against *B. fabae* as the days of incubation increased, where the highest inhibition was 68%. *Bacillus* and *Serratia* spp. were relatively effective rhizobacteria compared to other species in showing better inhibitory activity against *B. fabae*. The *Bacillus* spp. showed the PIRG that ranged from 10-68% of inhibition of *B. fabae* upon 3-9 days of incubation. Samuel Sahile et al. (2009) have reported 23-64% inhibition of this pathogen by *Bacillus* spp. obtained from faba bean leaves after 7 days of incubation. Ahmed (2015) and El-Banoby et al. (2013) have reported the inhibitory activity of 62.6% and 67.03% displayed by *B. subtilis*, respectively, upon 5 days of incubation, which is different from the finding of this study on the 5th days. The variation might be associated with the isolates sources, the inoculum size and or the incubation temperature used by the investigators.

Similarly, the different strains of *S. nematodiphila* exhibited the PIRG that ranged from 08-57% inhibition of *B. fabae*. *Serratia plymuthica* showed 51% inhibition of *Botrytis cinerea* (de Senna *et al*., 2017) and *S. marcescens* illustrated 54.81% inhibition against *R. solani* after 7 days of incubation (Ahmed, 2016). It is interesting that the dominant soil bacterium is represented by only one isolate *Pseudomonas* AAUB152 that displayed moderate inhibition ranging from 13% up to 53% upon 3-9 days of incubation which is contrary to the findings of Fekadu Alemu (2016) who showed that *P. fluorescens* isolates inhibited the mycelia growth of *B. fabae* by 88% on the 7th days of incubation. The different might be caused by the type of medium (King B) utilized by the author for the antagonistic assay, that favors the better performance of *P. fluorescens*. However, in the present study, 10% sucrose modified PDA was employed to study at the antagonistic activity of the bacterial isolates against *B. fabae*.

The isolates were further screened by using culture filtrates of different concentrations (Table 2.4). The results revealed the mycelial growth inhibition of *B. fabae* with better magnitude than observed in dual culture techniques. The culture filtrates of the isolates inhibited the test pathogen at concentrations of 5-20% that ranged from 6-82% (Table 2.4). The maximum inhibition of 27% was recorded from the culture filtrate of *B. subtilis* AAUB95 at 5% of the filtrate. In the dual culture assay, the isolate exhibited maximum antagonistic activity (68%) against the pathogen and displayed the highest inhibition of 82% at 20% concentration of its filtrate after 9 days of incubation. This may indicate more antibiotic producing ability of the isolate (*B. subtilis* AAUB95)

that can play role in the antagonism. *Serratia nematodiphila* AAUB146b was also effectively inhibited the test pathogen under *in vitro* conditions within the percentage of 21% at (5%) and 79% at (20%) concentrations.

Although, *B. tequilensis* AAUB100 was relatively weak at lower concentration (5%) compared to the two isolates, it displayed 80% inhibition at the treatment with 20% culture filtrate. In addition, *S. nematodiphila* AAUB146b (79%) was the best antagonistic of *B. fabae* next to *B. subtilis* AAUB95 (82%) and *B. tequilensis* AAUB100 (80%) at 20% of the filtrate. However, the culture filtrate of *S. marcescens*, suppressed the mycelial growth of *R. solani* by 65.6%, through the application of 100 µL after 7 days of incubation (El Khaldi *et al*., 2015). On the other hand, *Serratia* sp. C4 screened under *in vitro* conditions for its antifungal activity against *Fusarium oxysporum* f. sp. *lycoperscisi* (FOL) and led to 19.52% decreased in the radial growth under *in vitro* conditions and its culture filtrate supplemented to PDA medium at 20% (v/v) had lowered pathogen growth by 23% at 20% of concentration on the 5^{th} day of incubation at 25°C (Aydi-Ben Abdallah *et al*., 2017).

Table 2.4: Percent inhibition of radial growth (PIRG) displayed by the antagonistic rhizobacterial isolates against *Botrytis fabae* using different culture filtrate concentrations (5-20%)

Species	Inhibition over control at 5-20%v/v of culture filtrate			
	5%	**10%**	**15%**	**20%**
B. tequilensis AAUB100	15^{cdef}	30 ^{cde}	52 ^{abcdef}	80^{a}
S. nematodiphila AAUB113b	15 ^{cdef}	27 ^{cdef}	54^{abcd}	75^{abc}
S. nematodiphila AAUB146b	21^{abc}	41^{abc}	60^{abc}	79 ^{a}
B. subtilis AAUB92	13^{cdefg}	31 ^{cde}	53^{abcde}	73 ^{abcd}
S. nematodiphila AAUB113c	13 ^{cdefg}	24^{def}	39 ^{efg}	63^{defg}
E. hormaechei AAUB53	18^{bcd}	38^{abcd}	47 ^{cdef}	53 ^{gh}
B. macroides AAUB122a	26^{a}	31 ^{bcde}	46 ^{cdef}	66 ^{bcde}
B. subtilis AAUB122b	17^{cde}	41 ^{abc}	59^{abcd}	77^{ab}
S. nematodiphila AAUB115	09^{fg}	35^{bcd}	64^{ab}	65^{cdef}
E. hormaechei AAUB94	11^{efg}	20^{ef}	47 ^{cdef}	77 ^{ab}
B. subtilis AAUB95	27 ^{a}	47^{ab}	65^{ab}	82^{a}
S. nematodiphila AAUB77	17 ^{cde}	50^{a}	66^{a}	75 ^{abc}
E. hormaechei AAUB55	16^{cdef}	31^{cde}	62^{ab}	66 ^{bcde}
Pseudomonas sp. AAUB152	16 ^{cdef}	27 ^{cdef}	50 ^{bcdef}	72 ^{abcd}
A. johnsonii AAUB130b	25^{ab}	32 ^{bcde}	38^{efg}	42 ^{h}
A. johnsonii AAUB130a	06^{fg}	32 ^{bcde}	38 ^{efg}	59 ^{efg}
E. faecalis AAUB47	09 ^{fg}	15^{f}	27^{g}	29^{i}
A. nicotinovorans AAUB146a	26^{ab}	40 ^{abc}	48^{cdef}	61 ^{efg}
Bacillus sp. AAUB150	19^{bcd}	33^{bcde}	51^{bcdef}	63 ^{defg}
Bacillus sp. AAUB113b3	16 ^{cdef}	27 ^{cdef}	46 ^{cdef}	55^{fg}
CV	0.16	0.12	0.15	0.18

Mean values in the same column labeled with the same letter(s) as superscript are not significantly different ($p>0.05$) by Tukey HSD analysis of One-Way ANOVA. CV-indicates the coefficient of variation among means in the same column.

The antagonistic bacterial isolates were further characterized for their mechanism of antagonism and plant growth-promoting features (Table 2.5). The qualitative assay demonstrated that 75 and 60% of the isolates were positive for protease and lipase, respectively. A 100% and 75% of the *Bacillus* spp. were positive for protease and lipase production, respectively. In addition, HCN was produced by *Pseudomonas* sp. AAUB152, *B. macroides* AAUB122a and *B. subtilis* AAUB95, that showed maximum inhibition. *Bacillus* spp. that antagonized *F. oxysporum* was found to produced HCN (Kaur *et al*., 2018). *Serratia nematodiphila* AAUB113b and *S. nematodiphila* AAUB115 produced HCN with variable quantity of IAA. The production of IAA and HCN by *S. nematodiphila* NII-0928 was indicated by Dastager et al. (2010). Therefore, variability in the inhibition potential of the pathogen among the isolate was attributable to the antagonistic mechanisms such as the synthesis of lytic enzymes (protease and lipase), beside HCN synthesis. Although the antagonistic potential of *A. nicotinovorans* AAUB146a was improved from 15-27% of dual assay to 26-61% using the culture filtrate of different concentration, the isolate did not produce either the volatile metabolites (HCN and ammonia) and enzymes. This indicates as the antagonistic mechanisms employed by the strain might be other antagonistic property investigated in this study.

Table 2.5: Plant growth-promoting and antagonistic properties of rhizobacterial isolates from faba bean (*Vicia faba* L.) rhizosphere grown in Arsi and Bale Zones

Species	HCN	IAA (µg mL^{-1})	Ammonia	Protease	Lipase
Control	-	0.00^{j}	-	-	-
B. tequilensis 100 AAUB	-	-	+	+	+
S. nematodiphila AAUB113b	+	1.19^{ghi}	-	+	+
S. nematodiphila AAUB146b	-	-	+	+	+
B. subtilis AAUB92	+	15.51^{a}	+	+	-
S. nematodiphila AAUB113c	-	-	+	+	+
E. hormaechei AAUB53	-	8.12^{f}	-	+	-
B. macroides AAUB122a	+	13.93^{b}	+	+	+
B. subtilis AAUB122b	-	9.13^{ef}	+	+	+
S. nematodiphila AAUB115	+	0.27^{hi}	+	-	+
E. hormaechei AAUB94	-	0.18^{hi}	-	+	-
B. subtilis AAUB95	+	10.52^{cd}	+	+	+
S. nematodiphila AAUB77	-	11.62^{c}	-	-	+
E. hormaechei AAUB55	-	1.21^{ghi}	+	-	-
Pseudomonas sp. AAUB152	+	-	+	+	+
A. johnsonii AAUB130b	-	-	-	-	+
A. johnsonii AAUB130a	+	-	+	+	-
E. faecalis AAUB47	-	-	-	+	-
A. nicotinovorans AAUB146a	-	2.11^{g}	-	-	-
Bacillus sp. AAUB150	+	1.32^{gh}	+	+	-
Bacillus sp. AAUB113b3	-	10.21^{de}	-	+	+
CV		0.08	-	-	-

Mean values in the same column labeled with the same letter(s) as superscript are not significantly different ($p>0.05$) by Student-Newman-Keuls (S-N-K) of One-way ANOVA. CV-indicates the coefficient of variation among means in the same column.

Furthermore, the bacterial isolates showing antagonism also produced IAA and ammonia with 65 and 60%, respectively. As presented in Table 2.5, an IAA that ranged from 0.18 to 15.51 µg mL^{-l} was quantified. Maximum IAA was produced by *B. subtilis* AAUB92 (15.51 µg mL^{-l}), followed by *B. macroides* AAUB122a (13.93 µg mL^{-l}), *S. nematodiphila* AAUB77 (11.62µg mL^{-l}) and *B. subtilis* AAUB95 (10.52 µg mL^{-l}). Nabti et al. (2013) have reported *B. licheniformis* and *Bacillus* sp. with best IAA producing ablility,78 and 101 mg L^{-l}, respectively, in addition to lytic enzyme synthesis and inorganic phosphate solubilization properties. Kaur et al. (2018) have reported IAA that varied from 5-24 µg mL^{-1} in different plant growth-promoting bacterial isolates, which is different from the present study. The variation might be caused by the amount of L- tryptophan (10 g L^{-1}) used by the authors as the precursor for the synthesis of IAA, nevertheless, in the present study 1g of L- tryptophan was utilized.

The different strains of *S. nematodiphila* produced IAA that varied from 0.27-11.62 µg mL^{-1}. An IAA concentration of 64.75 µg mL^{-1} and 56.60 µg mL^{-1} was produced by *S. marcescens* subsp. *marcescens* strain KB01 and *S. marcescens* subsp. *marcescens* strain KB05, respectively (Hasuty *et al*., 2017), that is different from the current study. This variation might be caused by difference in strains or the concentration of L- tryptophan used and/or the incubation temperature.

The quantitative assay of TCP solubilization demonstrated 310-760 µg mL^{-1} and 200-620 µg mL^{-1} of solubilized TCP on the 3rd and 6th days of incubation, respectively, indicating solubilization reduction after 3rd days (Table 2.6). The TCP solubilization showed 4.6-12.81 and 2.7-9.7 fold over the control on the 3rd and 6th days of inoculation, respectively. A similar trend of reduction in TCP solubilization was reported by Mullisa Jida et al. (2016) in PGPR that solubilize inorganic phosphate.

Table 2.6: Phosphate solubilization efficiency of the different rhizobacterial isolates from faba bean (*Vicia faba* L.) rhizosphere; the amount of phosphorus released (µg mL^{-1}) (NBRIP liquid) (from inorganic calcium phosphate based upon days (3-6) of incubation (for isolates with PSI >2) and change in the initial pH (pH_o=7) of the medium

Species	PVK (solid) PSI	NBRIP (liquid) (µg mL^{-1}) in days		change in pH (3-6 days)	
		Day 3	Day 6	Day 3	Day 6
Control	1.00^{e}	55^{c}	58^{e}	6.40^{a}	6.90^{a}
B. tequilensis 100 AAUB	2.67^{a}	760^{a}	600^{a}	5.00^{de}	5.44^{ef}
S. nematodiphila AAUB113b	2.21^{cd}	410^{b}	340^{c}	4.50^{f}	5.69^{de}
S. nematodiphila AAUB146b	2.24^{bcd}	650^{a}	440^{bc}	5.01^{de}	5.00^{f}
B. subtilis AAUB92	2.51^{abc}	75^{a}	600^{a}	5.33^{c}	5.49^{ef}
S. nematodiphila AAUB113c	2.16^{cd}	740^{a}	460^{b}	5.91^{b}	6.24^{bc}
B. macroides AAUB122a	2.64^{ab}	700^{a}	620^{a}	4.98^{de}	6.50^{ab}
B. subtilis AAUB122b	2.14^{cd}	650^{a}	450^{bc}	4.90^{de}	6.18^{bcd}
S. nematodiphila AAUB115	2.18^{cd}	600^{a}	440^{bc}	5.19^{cd}	6.06^{bcd}
B. subtilis AAUB95	2.09^{cd}	410^{b}	370^{bc}	5.18^{cd}	6.18^{bcd}
S. nematodiphila AAUB77	2.43^{abcd}	440^{b}	350^{bc}	4.94^{de}	5.75^{cde}
Pseudomonas sp. AAUB152	2.06^{cd}	310^{b}	200^{d}	5.00^{de}	5.44^{ef}
CV	0.12	0.15	0.10	0.18	0.22

Mean values in the same column labeled with the same letter(s) as superscript are not significantly different (p>0.05) by Tukey HSD analysis of One-way ANOVA. CV-indicates the coefficient of variation among means in the same column.

The change in the pH of NBRIP was related to TCP solubilization and the drop in pH value was seen from the 3rd-6th days. A reduction of pH from neutral to 4.5 was observed in *S. nematodiphila* AAUB113b on the 3rd days and a pH of 5 was displayed by *S. nematodiphila* AAUB146b on the 6th days. However, the solubilized phosphate by *S. nematodiphila* AAUB113b was lower (410 µg mL^{-1}) on the 3rd days and 440 µg mL^{-1} by *S. nematodiphila* AAUB146b on 6th days. This may imply as the drop of pH is not the only factor for TCP solubilization. Mullisa Jida et al. (2016) have reported similar results of pH reduction in phosphate solubilizing rhizobacteria as a functions of incubation days. Further, the correlation analysis between pH and TCP solubilization revealed an inverse (Table 2.7) relationship (r= -0.422*). Thus, this study pointed out the role of medium acidity in phosphate solubilization, as maximum concentration of solubilized TCP was obtained at the time of pH reduction from neutral to acidic conditions (pH<7) on the 3rd days.

Table 2.7: The Pearson`s correlation analysis between Phosphate solubilization and the change of pH in the medium

Correlations

		solubilized phosphate	pH change
solubilized phosphate	Pearson Correlation	1	-.422*
	Sig. (2-tailed)		.040
	N	24	24
pH. change	Pearson Correlation	-.422*	1
	Sig. (2-tailed)	.040	
	N	24	24

*. Correlation is significant at the 0.05 level (2-tailed).

2.10 Conclusion and Recommendations

This study pointed out *Bacillus* and *Serratia* species as potential isolate in showing the best inhibitory activity against *Botrytis fabae* along with the antagonistic and plant growth-promoting traits. Of the *Bacillus* strains, *B. subtilis* AAUB95 showed the best antagonistic feature against *B. fabae* with plant growth-promoting traits production and thus can be utilized as biofungicides either under greenhouse and or field conditions after testing other inoculant traits.

Therefore, based on this findings, it could be recommended that the isolates can be utilized as biofertilizers and bioprotectant of plants either under greenhouse and or field conditions by combining together with other beneficial rhizosphere microorganisms such as *Trichoderma* spp.

CHAPTER 3

Isolation and Characterization of *Trichoderma* spp. from the rhizosphere of faba bean (*Vicia faba* L.) and for their antagonistic effect against *Botrytis fabae* and plant growth-promotion under *in vitro* conditions

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Abstract

Trichoderma is one of a filamentous fungus that can acts as biological control agent of several fungal pathogens of plants. Chocolate spot is one of the emerging fungal disease of faba bean caused by *Botrytis fabae*. This study was conducted to characterize *Trichoderma* spp. for their antagonistic properties against *B. fabae* and plant growth-promoting traits. The data were analyzed by SPSS version 24. The isolates of *Trichoderma* inhibited the growth of *B. fabae* from 7-88% upon 3-9 days of incubation using dual culture method. Isolate AAUT21 and AAUT14 were the most effective ones with 85 and 88% of inhibition. Inhibition over the control was increased from 13-64% upon 3-9 days using sealed plate method. AAUT14, AAUT44 and AAUT45 allowed the least level (< 3.0 scale) of *B. fabae* infection in Ashebeka and Hachalu varieties of faba bean in the detached leaf assay with significant difference from the controls ($p<0.05$). Moreover, 63%, and 95% of the isolate were positive for IAA and ammonia synthesis, respectively. Different *Trichoderma* isolates solubilized tricalcium phosphate (TCP) that ranged from 135-575 µg mL^{-1} upon 3-6 days. Isolate AAUT6 released 338 µg mL^{-1} of solubilized TCP followed by AAUT14 (327 µg mL^{-1}) and AAUT4 (326 µg mL^{-1}) during the 3rd day of incubation. The Pearson`s correlation analysis indicated a negative correlation between pH and TCP solubilization (r=-0.612**). Based on the cultural characteristics and tef1α gene sequence analysis, isolate AAUT21, AAUT14, AAUT44, AAUT45, AAUT6, AAUT30, and AAUT4 were identified as *T.*

afroharzianum AAUT21, *T. harzianum* AAUT14, *T. tomentosum* AAUT44, *T. afroharzianum* AAUT45, *T. harzianum* AAUT6, *T. afroharzianum* AAUT30 and *T. tomentosum* AAUT4, respectively. *Trichoderma harzianum* AAUT14 showed the best antagonistic feature against *B. fabae* along with different plant growth-promoting traits. Thus, the strain can be a candidate to be further evaluated as biofungicide of *B. fabae* under greenhouse conditions.

Keywords: Dual assay; inhibition; mycoparasitism; rhizosphere

3.1 Introduction

Trichoderma species are cosmopolitan fungi that are present in all types of soil and other habitats (wood decay, barks etc.). *Trichoderma harzianum, T. koningii, T. orientale, T. tomentosum* and *T. viride* are usually found in cellulose rich soil and other environments (Jang *et al*., 2017). They function as parasites and antagonists of plant pathogenic fungi. Thus, they are effective in controlling phytopathogens due to their ability to grow toward the hyphae of other fungi, coil around them and degrade the cell wall of the pathogen.

These biocontrol fungi acquire different direct and indirect mechanisms to inhibit aerial and root pathogens (myco-parasitism). Mycoparasitism is attributed to the synthesis of lytic enzymes such as chitinases, glucanases and protease that are involved in cell wall degradation of the fungal pathogens (Kapteyn *et al*., 1996). *Trichoderma* species are also known in producing a plethora of secondary volatile and non-volatile metabolites with inhibitory activity against the diverse groups of pathogenic microorganisms (Reino *et al*., 2008). They compete out the pathogens in nutrient utilization that limits the germination of conidia and germ tube development, thereby reducing the number of infection sites, and aggressively colonize the points of entry and prevents the introduction of pathogens into host (Card *et al*., 2009).

This fungus is a good biological control agents (BCAs) of various aerial, root and seed diseases of economically important crops (Galarza *et al*., 2015; Reddy *et al*., 2018). Elad (2000) has reported that about 90% different strains of *Trichoderma* spp. are being used as BCAs. In addition, *Trichoderma* spp. are capable of producing ammonia and hydrogen cyanide to suppress at the efficiency of pathogens to infect plants (Keszler *et al*., 2000) and acquire multiple plant growth promoting properties that involve in phosphate solubilization, and phytohormones production. According to Reino et al. (2008), *T. harzianum, T. hamatum, T. asperellum* and *T. atroviride* are commercialized for the control of phytopathogens and plant growth promoters in agriculture.

Chocolate spot disease is one of the emerging fungal disease caused by *Botrytis fabae* (*cinerea*) with a wide host range of vegetables and legumes. The disease is widely distributed in Tunisia, Algeria, Morocco, Libya, Ethiopia and other countries such as Spain, Norway, Germany, Scotland,

Russia, Japan, China, Canada and Australia (Hebblethwaite, 1983). Several studies showed that the disease can be managed through integrated approach including careful selection of seeds, the use of resistant varieties, and applications of fungicides such as MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80WP (Mancozeb), (Dagne Kora *et al*., 2017; Addisu Tegegn *et al*., 2019).

Even though fungicides are effective in controlling fungal pathogens of plants, they induce resistance to the pathogen (Hassan *et al*., 2006). In order to minimize the use of synthetic fungicides, selected microorganisms can be used as part of the integrated pest management (IPM). Although studies on the antagonistic properties of *Trichoderma* spp, *T. harzianum*, *T. viride* and *T. virens* on different fungal diseases showed disease reduction by 24% up to 98% under greenhouse and field conditions on vegetables (Bokhari and Perveen, 2012; Kator *et al*., 2015), chickpea (Yadav *et al*., 2011; Subhani *et al.*, 2013), potato blight (Al-Mughrabi, 2008; Quiroga-Rojas et al. 2012). However, there is limited report on chocolate spot disease on faba bean.

To that end, some studies showed the antagonistic fungi, *T. album* (Barakat *et al.*, 2014), *Trichoderma* spp. (Ermias Teshome *et al.*, 2013) reduced the disease incidence of chocolate spot by 78% and 51%, respectively. Mbazia et al. (2016) have also showed 35% reduction in chocolate spot infestation on leaves of faba beans by *T. viride.* The application of *Trichoderma* spp. showed an antagonistic efficacy ranging from 47.6 to 98% against *B. fabae* (Samuel Sahile *et al*., 2011).

Thus, the objective of this study was to isolate, screen, identify and characterize *Trichoderma* spp. for their multiple plant growth-promoting and antagonistic properties against *B. fabae*, the causative agent of chocolate spot in faba bean.

3.2 MATERIALS AND METHODS

3.2.1 Description of the study Areas

Soil samples were collected from Arsi zone (at DigelunaTijo and Tiyo districts) and Bale zone (Goba district). DigelunaTijo is located at geographical coordinates of 7° 19' 60.00" N latitude and 39° 14' 60.00" E longitude and altitude between 2500-3560 meter above sea level (a.s.l). The district is found in Arsi zone, bordered by Bekoji on the South, Munesa on the South-West, Tiyo on the North-West, Hitosa on the North, Tena on the North-East and Sherka on the East. It has 39.5% arable, 27.4% pasture, 13.3% forest and 19.8% as swampy lands (SEP, 2019). Tiyo district is found at 7° 49' 59.99" N latitude and 39° 09' 60.00" E longitudes, altitude of 1780-3100 m.a.s.l. The district is bordered by Munesa on the South, Ziway Dugda on the West, Hitosa on the North-East and Digeluna Tijo district in the South-Eastern part. This district has 40% arable, 23.1% pasture, 8.7% forest and 28.2% as a swampy land (SEP, 2019) and altitude of 1780-3100 m. a. s. l, with a temperature of 5-28°C and relative humidity of 43-60%. Both DigelunaTijo and Tiyo districts have bimodal rainfall having March to April short rainy and July to October, long rainy seasons.

Goba district is found in Oromia region, Bale zone. It is bordered on the South by Mennana Harena Buluk, on the West by West Arsi, on the North by Mena River and on the South-East by Berbere town with coordinates of 6° 49' 59.99" N latitude and 39° 49' 59.99" E longitude. This district is located at a distance of 14km South of Bale zonal town, Robe at 444km South-East of Addis Ababa, with coordinates of 6° 49' 59.99" N latitude and 39° 49' 59.99" E longitude. The altitudes of this district is 1500 - 4377 m.a.s.l, having a temperature of 0-23°C (BZMED, 2007). As a part of Bale zone, Goba District has two types of rainfall regime. The long rainy season extends from March to April with high rain fall during June, July and August. The second rainy season of rain fall regime is influenced by equatorial westerly and easterly winds with rainfall during spring and autumn. The major soil types are Chromic and Pellic Vertisols in some parts, Chromic, Orthic and Vertic Luvisols around highlands and plateaus areas. A survey of the land in this District shows that 13% is arable or cultivable, 27.6% pasture, 54.6% forest (or part of the Bale Mountains

National Park), and the remaining 4.8% is considered degraded or otherwise unusable (BOARD, 2012).

For this study, Arsi and Bale zonal area were selected because the symptom of chocolate spot was prevalently observed on faba bean crops under field conditions, indicating the presence of *Botrytis* spp. (*B. fabae* and *B. cinerea*) in the areas (Figure 2.1). These sampling districts were selected because the standing faba bean crops were severely infected by chocolate spot disease (Dagne Kora *et al*., 2017; Shiferaw Deneke *et al*., 2018).

3.2.2 Soil Sampling and Processing

Rhizospheric soil samples were collected from the study areas during faba bean growing season of 2016. The samples were coded as FRSS-01, FRSS-02, FRSS-03, FRSS-04, FRSS-05, FRSS-06, FRSS-07, FRSS-08, FRSS-09, FRSS-10, FRSS-11, FRSS-12, FRSS-13, FRSS-14, FRSS-15 and FRSS-16. "FRSS"- stands for faba bean rhizosphere soil samples with corresponding number. From each sampling site, faba bean plants were uprooted and about 50 g of root adhering soil samples were collected from the standing healthy faba bean plants, in triplicates, and composited in one and collected in alcohol-surface sterilized polyethylene bags and stored at room temperature for the isolation of rhizosphere bacterial species.

3.2.3 Isolation and cultural characterization of *Trichoderma* Isolates

Trichoderma spp. were isolated according to Elad et al. (1998). A 10 g of each soil sample was dissolved in 90 mL sterilized-water in a 250 mL flask. The suspension was prepared to appropriate dilutions from which 0.1 mL of the final dilution was transferred onto Rose Bengal agar (RBA) medium ($MgSO_4.7H_2O$, 0.20 g/L, K_2HPO_4, 0.9 g/L, KCl, 0.15 g/L, NH_4NO_3, 1 g/L, glucose, 3 g/L, rose Bengal, 0.15 g/L and agar, 20 g/L). The plates were incubated at 25 ± 2°C for 7-10 days. Typical *Trichoderma* resembling colonies were picked according to Barnnet et al. (1972) and sub-cultured on potato dextrose agar (PDA) plates for purification and characterization. The purified cultures were then compared with characteristics and illustrations on the manual to confirm the isolates, and stored in PDA slants at 4°C for further study.

3.2.4 Isolation of the Test pathogen (*Botrytis fabae*)

The test fungus was isolated from leaves (showing symptoms of chocolate spot) of faba bean collected from the study areas and seeds from storage according to Shinde (2016) using faba bean dextrose agar (FDA) medium (200 g faba bean seed extracts, 20 g dextrose, and 18 g agar). Seeds and leaves were washed with tap water, surface sterilized with 3% sodium hypochlorite for 5 min and rinsed in sterilized-distilled water and air dried. Four to six seeds and leaf cuts per plate were transferred to FDA medium and incubated at room temperature for 10-12 days under 12 hrs. light and 12 hrs. dark conditions and purification was done by sub culturing on PDA plates. The identification of *B. fabae* was conducted through looking at the cultural characteristics (i.e. colony color and formation of sclerotia on the PDA). The purified culture was then preserved at 4°C using PDA slants.

3.2.5 Pathogenecity test of the test pathogen (*Botrytis fabae*)

The pathogenecity test was done according to Abdel-Aleem et al. (2011). Faba bean seeds were surface sterilized with 3% sodium hypochlorite for 5 min and rinsed in sterilized-distilled water. Faba bean seeds were sown in 3 kg alchol sterilized pots filled with sterile soil. Fifteen days after planting, 12 mL of the spore suspension was adjusted to $4.5x10^5$ spores/mL using haemocytometer, sprayed on the foliar parts of faba bean seedlings and covered with sterilized plastics, whereas the control was sprayed with 12 mL sterilized water. The occurrence of chocolate spot diseases infection on faba bean seedlings was checked interms of its intensity and severity visually and the infected plants parts were cut and used to re-isolate the pathogen after 7^{th} days of spraying to confirm the Koch's postulate. The isolates were tested for cultural characteristics and compared with the reference strain of *B. fabae* previously isolated from faba bean and obtained from Phytopathology Laboratory of the Ethiopian Institute of Agricultural Research (EIAR).

3.3 Cultural Characterization and Identification of the *Trichoderma* Isolates

3.3.1 Cultural characterization

The *Trichoderma* isolates were grown on potato dextrose agar (PDA) medium at 25°C for 5-7 days. They were examined for their colony color both on the front and reverse side of the plate, and compared with morphological keys described by Barnnet et al. (1972) and identified to the genus level.

3.3.2 Molecular Identification of the *Trichoderma* Isolates

3.3.2.1 Extraction of DNA

The DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) Method according to Doyle and Doyle (1987) for all *Trichoderma* resembling isolates determined under cultural characterization. Three hundred milligrams (300 mg) of mycelia mat were transferred to sterile pestle and mortar and grounded using lysis buffer (100 mM Tris HCl [pH8.0], 50 mM EDTA, 3% SDS), and transferred to 1.5 mL Eppendorff, with the addition of CTAB buffer, centrifuged at 13,000 rpm for 10 min. The supernatant was then mixed with 210 mg/L of RNase A and incubated at 37°C for 15 min, to which chloroform: Isoamyl alcohol (25:24:1) was added and centrifuged at 13,000 rpm for 10 min. The upper aqueous layer was treated with 100% cold ethanol, precipitated at -20°C for 30 min, and centrifuged at 12,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min. The DNA pellets were air dried and suspended in 1×TE buffer. For PCR reactions, 1µL of the extracted DNA was utilized as a template for the amplification.

3.3.3 Quantity and Quality Determination of DNA

The quantity of the extracted genomic DNA was determined by measuring the absorbance at 260 nm using Thermo Scientific Nano Drop (NanoDrop 2000). The quality and suitability for PCR reaction of the DNA was checked by visual comparison with the standard DNA size markers after

electrophoresis through 0.8% agarose TAE (Tris-acetate EDTA) gels stained with 0.5 mg mL^{-1} ethidium bromide (Sigma Chemicals Co.).

3.3.4 Primers and PCR Conditions for DNA (Tef1α) gene Amplification

The amplification of 1.3kb translation elongation factor one alpha (tef1α) gene was performed using the primer pairs of EF1728F (5`-CATCGAGAAGTTCGAGAAGG-3`) (Chaverri and Samuels, 2003) and TEF1LLErev (5`-AACTTGCAGGCAATGTGG-3`) (Jaklitsch *et al*., 2005). The total volume of PCR reaction used was 50 µL. The PCR amplifications were performed with 20 µL (Gotaq polymerase), 1.12 µL forward primer, 1.12 µL reverse primer, 0.80µL (bovine serine albumin, 14.96 µL of nuclease-free water and 12 µL of the DNA template (Table 3.1). The PCR cycling conditions consisted of an initial denatuartion step of 95°C for 2 min and subjected to 35 cycles of the following program 95°C for 45s, 58°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were visualized by ethidium bromide staining on 1.5% agarose gel electrophoresis and purified according to the gel extraction (Nucleo Spin Extract II, Macherey-Nagel, Düren, Germany) clean-up kit protocol and sequencing was performed at Macrogen Inc. (Seoul, Korea).

Table 3.1: Components and amounts of PCR mixtures used for DNA amplifications of the *Trichoderma* isolates

S.N	Components	Amount (µL)
1	Gotaq polymerase	20.00
2	Primer 1 (EF1728F)	1.12
3	Primer 2 (TEF1LLErev)	1.12
4	Bovine serine albumin (BSA)	0.80
5	ddH_2O	14.96
6	DNA template	12.00
Total volume of reaction		**50.00**

3.3.5 Sequence Analysis (BLAST) and Identification

The Tef1α gene sequences were subjected to the BLAST search program (National Center for Biotechnology Information) to find a similarity index between sequences. Each sequence was subjected to an individual BLAST search to be verified in Gene Bank. The BLASTn similarity search program was used to find homologous sequence against the NCBI nucleotide database that confirmed the species level similarity with the query sequence of the isolates.

3.4 Screening of *Trichoderma* Isolates for the antagonistic Property

3.4.1 Dual Culture Assay

The *Trichoderma* isolates were screened for their biological control potential of *B. fabae* using dual culture assay according to Dennis and Webster (1971). In this method, 5 mm cut of mycelial agar disc from 5 days' culture of *Trichoderma* spp. was cut and placed on one side of PDA contained in 90 mm diameter petri plate. After 24 hrs. of incubation 5 mm mycelial agar was cut from the margin of 7 days old culture of *B. fabae* and placed on the same plate at the opposite side. The plates were incubated at 25±2ºC until the control was fully covered with the growth of *B. fabae*. The antagonistic activity and percent inhibition of radial growth (PIRG) were calculated according to Morton and Stroube (1955). The radial growth of *B. fabae* against *Trichoderma* isolate was taken using measuring ruler in millimeter (mm) on the 3rd, 5th, 7th and 9th days of incubation.

$PIRG = R_1\text{-}R_2/R_1X100\%$

Where, PIRG- percent inhibition of radial growth, R_1-indicates the radius of *B. fabae* in the control and R_2-radial growth of *B. fabae* toward the *Trichoderma* isolates in the treated Petri plates.

The status of antagonism between the *Trichoderma* isolates and *B. fabae* was tested following the method suggested by Bell et al. (1982), with ranking scales of 1-5. Where, (1)-*Trichoderma* isolates over grew *B. fabae* and covered above 80% of the medium, (2)- *Trichoderma* isolates over

grew *B. fabae* and covered at least 75% of the medium, (3)- *Trichoderma* isolates and *B. fabae* each colonized one half, 50% of the medium surface and neither microorganism appeared to dominate each other, (4)- *B. fabae* colonized at least two-third of the medium surface and (5)- *B. fabae* completely over grew *Trichoderma* isolates and occupied the entire surface of the medium.

3.4.2 Volatile Metabolite Production Assay

Volatile metabolite production was performed by sealed plate method as described by Mannaa and Kim (2018). From 7 days old culture of *Trichoderma* isolates, 5 mm diameter of mycelial cut was placed on the central part of PDA medium. At the same time, the center of another PDA plate containing the same size of *B. fabae* was prepared. The bottom of both plates were then sealed together with parafilm tape and incubated at 25±2^{o}C until the *B. fabae* was fully grown on the control PDA plates. The mycelial growth pattern of the test pathogen was measured in mm on the 3rd, 5th, 7th and 9th days of incubation. All the assays were performed in triplicates and the PIMG was calculated using the following formula (Mannaa and Kim, 2018).

PIMG = D_1-D_2/D_1X100, Where, PIMG- percent inhibition of mycelial growth, D_1-the diameter of *B. fabae* in the control plate and D_2- the diameter of *B. fabae* in the treated plate.

3.4.3 Detached Leaf Assay

The effect of five *Trichoderma* isolates on the level of *B. fabae* infection was evaluated using detached leaf assay according to Ermias Teshome et al. (2013). The faba bean leaves obtained from Ashebeka and Hachalu varieties were washed under tap water, surface sterilized with 1% sodium hypochlorite for 1 min, washed thoroughly with sterilized water, kept in sterilized glass plates and sprayed on their biaxial surface with small amount of water. The spore concentrations for *B. fabae* and *Trichoderma* species were adjusted to 4.5x10^{5} and 1.0x10^{8} spore mL^{-1}, respectively using haemocytometer. The leaf samples were then treated with 3 drops of spore concentration of *B. fabae* pretreated with *Trichoderma* isolates one day before the inoculation of the test pathogen. The controls were treated with pathogen only. All the assays were undertaken in three replications and incubated for five days. The severity of the infection on the leaf samples

was rated according to Fotopoulos (2008) using scale (0-3). Where, 0= no lesion, 1= flecked lesion, 2=limited lesion and 3=spreading lesion.

3.4.4 Mycoparasitism

The *Trichoderma* isolates were tested for their ability to parasitize *B. fabae* using the slide culture technique according to Soliman et al. (2016). A grain size (approx. 2x2 mm) of *Trichoderma* isolates and *B. fabae* was grown on the same glass slide containing the PDA blocks (10x20 mm) side by side and incubated at 25±2^{o}C for 72 hrs. After incubation, the slides were observed and photographed under microscope (OLYMPUS-BX51, Germany) at 400x to check the growth and the presence of hyphal coiling over *B. fabae* and compared to the control.

3.5 Plant growth-promoting Characteristics of the *Trichoderma* Isolates

3.5.1 Qualitative and Quantitative Tricalcium phosphate (TCP) Solubilization

Nineteen (19) isolates were tested for their capacity to solubilize inorganic phosphate was on Pikovskaya's (PVK) agar medium using TCP as the sole source of phosphate and the mobilize phosphate was determined according to Nautiyal (1999) quantitatively, using National Botanical Research Institute Phosphate (NBRIP) medium. The medium contained; (g/L), glucose (10.0), $Ca_3(PO_4)_2$ (5.0), $MgCl_2.6H_2O$ (5.0), $MgSO_4.7H_2O$ (0.25), KCl (0.2) and $(NH)_2SO_4$ (0.1).

For quantification, cultures were taken after 3, 6, and 9 days of incubation and centrifuged (Centrifuge, Wagtech international, United Kingdom) at 10,000 rpm for 25 min. The pH change of the supernatant was measured using digital pH Meter (NIG 333, New Delhi, India). The amount of phosphate was detected using Olsen, s Method. One mL of the supernatant was taken and quantified at 880 nm using spectrophotometer (6405UV/Vis., Jenway, England) and the concentration was calculated from the standard curve constructed from known quantities of potassium di-hydrogen orthophosphate, KH_2PO_4 (µg mL^{-1}).

3.5.2 Qualitative and Quantitative Assay of Indole-3- Acetic Acid (IAA)

Nineteen (19) isolates ability to produce IAA was checked qualitatively as described by Hartmann et al. (1983). Spores were extracted from 5 mm mycelial cut of 5-days grown *Trichoderma* isolates ($1x10^6$ spores/mL) and inoculated in 50 mL potato dextrose broth (PDB) amended with L-tryptophan (1g L^{-1}) in 250 Erlenmeyer flasks and incubated at 25±2°C on an orbital shaker (ZJZD-III, Shanghai, China) at 125 rpm for 72hrs. Uninoculated flasks were included as control. The cultures were centrifuged (Wagtech international, United Kingdom) at 3000 rpm for 30 min, from which 2 mL of the supernatants were mixed with two drops of orthophosphoric acid and 4 mL of Salkowaski`s reagent (50 mL of 70% per chloric acid and 1 mL of 0.5 M $FeCl_3$ and 49 mL sterilized distilled water), and incubated at room temperature in dark for 30 min. The development of pink colour was visually checked as an indicator of IAA production and quantified at 530nm. The concentration of IAA produced was calculated in µg mL^{-1} using the standard curve sketched from known concentrations of IAA. the standard curve was made using nutrient and potato dextrose broth medium amended with tryptophan at 0, 20, 40, 60, 80 and 100 (µg/mL) using acetone as a solvent of standard solutions preparation (Gordon & Weber, 1951).

3.5.3 Qualitative Assay of Ammonia Production

The qualitative assay of ammonia production by *Trichoderma* (19 isolates) was done according to Cappuccino and Sherman (1992). The *Trichoderma* isolates were grown in 5 mL peptone water at 28±2°C for 4 days. Following the incubation, 1mL of Nessler's reagent ($HgCl_2$, 10.0 g, KI, 7.0g, NaOH, 16.0 g, H_2O (ammonia free), 100mL)) was added to the cultures to detect the development of yellow colour as an indicator of ammonia production.

3.6 Data Analysis

The statistical analysis was performed by One-Way ANOVA of SPSS version 24. The comparisons among means were done by using Tukey HSD and Student-Newman-Keuls (S-N-K) analysis at $\alpha = 0.05$. Values were considered significant at $p<0.05$.

3.7 Results and Discussion

A total of 19 *Trichoderma* isolates were isolated and characterized on the basis of their cultural and genetic features (Table 3.2). The colonies were whitish in color on the PDA medium at the beginning on the front side and changed to green as they got older within 3-5 days with small and whitish in color at 25±2°C incubation for 48-72hr. However, following sub-culturing onto PDA, the color was changed to greenish indicating variability (change in colour) as a function of time. The edge of cultures remains white; whereas the reverse side of the plates appeared yellowish on PDA, the typical characteristics of *Trichoderma* spp. The data showed that 58, 21 and 21% of the mycelial color of the isolates on the front side of PDA whitish green, pale green and deep green, and the same percentage of the reverse side of the cultures was dull yellowish, pale yellow and yellow colour, respectively (Table 3.2).

The cultural characteristics together with translational elongation factor 1 alpha gene (Tef1α) sequence analysis on NCBI, the isolates were identified into five closest species of *Trichoderma afroharzianum*, *Trichoderma harzianum*, *Trichoderma tomentosum*, *Trichoderma* spp. and *Trichoderma orientale* (Table 3.2). Translational elongation factor 1 alpha gene (Tef1α) sequence was used for the identification of *Trichoderma* isolates in this study, because this gene has a unique genetic resolution and has a good sequence variation to be used in fungal taxonomy and species differentiation which is also proven to be useful in studying the *Trichoderma* genera (Zhao *et al*., 2011; Alhawatema *et al*., 2019). The relative abundance of the species showed that 42% of the isolates were *Trichoderma afroharzianum*, 21% *Trichoderma tomentosum*, 16% *Trichoderma harzianum*, 16% *Trichoderma* sp. LSBA1. and 5% *Trichoderma orientale*. The percentage sequence similarity of each isolate is given below (Table 3.2). All the identified isolates are preserved at Addis Ababa University in the applied microbiology laboratory and University of Lavras, culture collection center, Brazil.

Table 3.2: The Identification of *Trichoderma* isolates from the rhizosphere of faba bean collected from Arsi and Bale zones

Isolates	Cultural characteristics (color)		Nearest species from GenBank (Tef1*αg*)	Similarity (%)	Accession Number of the nearest spp.	Identified species
	Front	Reverse				
AAUT21	Whitish green	Dull yellowish	*Trichoderma afroharzianum* LESF553	99.60	KT279014.1	*T. afroharzianum* AAUT21
AAUT14	Whitish green	Dull yellowish	*Trichoderma harzianum* T2018	98.90	MG735712.1	*T. harzianum* AAUT14
AAUT3	Pale green	Pale yellow	*Trichoderma tomentosum* MIAE00032	98.40	HM176580.1	*T. tomentosum* AAUT3
AAUT4	Pale green	Pale yellow	*Trichoderma tomentosum* MIAE00032	98.40	HM176580.1	*T. tomentosum* AAUT4
AAUT35	Whitish green	Dull yellowish	*Trichoderma afroharzianum* CNFG3201	99.20	MH662568.1	*T. afroharzianum* AAUT35
AAUT6	Whitish green	Dull yellowish	*Trichoderma harzianum* T2018	98.90	MG735712.1	*T. harzianum* AAUT6
AAUT8	Whitish green	Dull yellowish	*Trichoderma harzianum* T2018	98.90	MG735712.1	*T. harzianum* AAUT8
AAUT19	Whitish green	Dull yellowish	*Trichoderma afroharzianum* KUC21406	99.20	MN580168.1	*T. afroharzianum* AAUT19
AAUT10	Deep green	Yellow	*Trichoderma* sp. LSBA1	98.20	KP743134.1	*Trichoderma* sp. AAUT10
AAUT16	Pale green	Pale yellow	*Trichoderma tomentosum* MIAE00032	98.40	HM176580.1	*T. tomentosum* AAUT16
AAUT17	Deep green	Yellow	*Trichoderma orientale* PPRI 3894	98.80	EU401579.1	*T. orientale* AAUT17
AAUT30	Whitish green	Dull yellowish	*Trichoderma afroharzianum* LESF553	99.60	KT279014.1	*T. afroharzianum* AAUT30
AAUT37	Whitish green	Dull yellowish	*Trichoderma afroharzianum* LESF553	99.60	KT279014.1	*T. afroharzianum* AAUT37
AAUT38	Whitish green	Dull yellowish	*Trichoderma afroharzianum* LESF553	99.60	KT279014.1	*T. afroharzianum* AAUT38
AAUT39	Deep green	Yellow	*Trichoderma* sp. LSBA1	98.20	KP743134.1	*Trichoderma* sp. AAUT39
AAUT40	Deep green	Yellow	*Trichoderma* sp. LSBA1	99.20	KP743134.1	*Trichoderma* sp. AAUT40
AAUT44	Pale green	Pale yellow	*Trichoderma tomentosum* MIAE00032	98.40	HM176580.1	*T. tomentosum* AAUT44
AAUT50	Whitish green	Dull yellowish	*Trichoderma afroharzianum* LESF553	99.60	KT279014.1	*T. afroharzianum* AAUT50
AAUT45	Whitish green	Dull yellowish	*Trichoderma afroharzianum* LESF553	99.60	KT279014.1	*T. afroharzianum* AAUT45

The effectiveness of the *Trichoderma* isolates to inhibit the radial mycelial growth of the test pathogen *B. fabae* varied from 7% up to 88% upon 3-9 days using dual culture assay (Table 3.3). Thus, the inhibitory activity of the isolates was within the range of 7-59% and 53-88% upon 3 and 9 days of incubation. Among the isolates, *T. afroharzianum* AAUT21 and *T. harzianum* AAUT14 were the most effective ones with 58-88% PIRG at all incubation, whereas *T. tomentosum* AAUT3, *T. tomentosum* AAUT4, *T. harzianum* AAUT6, *T. harzianum* AAUT8, and *T. afroharzianum* AAUT19 were weak at the beginning but showed the maximum inhibition of 80% after 9 days of incubation. It is interesting to note that *T. afroharzianum* AAUT45 showed PIRG of 10% with 3 days of incubation but increased 8-fold (81%) after 9 days of incubation. All these strains fell within the best Bell's (1982) scoring scales (Table 3.3). However, *T. orientale* qualified to the moderate inhibition scoring.

The isolates also showed inhibitory activity of 35-72% over the control which was similar to 45-78% inhibitory activities of different *Trichoderma* spp. against *B. fabae* after 7 days of incubation over the control as reported by Bogumił et al. (2013). On the contrary, Barakat et al. (2014) have reported that *Trichoderma* spp. inhibited the mycelial growth of *B. fabae* by 51-78% after 6 days of incubation. In Ethiopia, Samuel Sahile et al. (2011) have showed the most effective *Trichoderma* spp. obtained from faba bean phylloplane inhibited *B. fabae* by 48-98% within a short incubation time of 3 days. The authors argued that effectiveness is affected by different factors such as source of the isolates, difference of the species, the metabolites released by the different strains, and or the way by which an inhibition was determined and the days of incubation. Based on Bell`s scale, 75% of the isolates covered the medium through over growing *B. fabae* compared to 21% of the isolates that failed to do so after 7 days of incubations in the present study.

Table 3.3: The effects of *Trichoderma* isolates on the inhibition of radial growth (%) of *Botrytis fabae* upon 3-9 days of incubation using dual culture assay

Sample	Identified species	Inhibition of radial growth (%) over control (days)				Bell's scale
		3rd days	5th days	7th days	9th days	
FRSS-13	*T. afroharzianum* AAUT21	58^{a}	61^{ab}	68^{ab}	85^{a}	1
FRSS-09	*T. harzianum* AAUT14	59^{a}	65^{a}	72^{a}	88^{a}	1
FRSS-03	*T. tomentosum* AAUT3	47^{b}	54^{abc}	59^{abc}	83^{a}	1
FRSS-02	*T. tomentosum* AAUT4	39^{bc}	46^{bcde}	51^{bcd}	81^{a}	1
FRSS-04	*T. afroharzianum* AAUT35	35^{cd}	42^{cdefg}	47^{cd}	74^{ab}	2
FRSS-01	*T.harzianum* AAUT6	43^{bc}	48^{bcd}	53^{abcd}	81^{a}	1
FRSS-15	*T.harzianum* AAUT8	42^{bc}	44^{bcde}	53^{abcd}	79^{ab}	2
FRSS-07	*T. afroharzianum* AAUT19	47^{b}	48^{bcd}	54^{abcd}	74^{ab}	2
FRSS-02	*Trichoderma* sp. AAUT10	40^{bc}	45^{bcde}	51^{bcd}	65^{ab}	2
FRSS-06	*T. tomentosum* AAUT16	10^{gh}	24^{h}	35^{d}	65^{ab}	2
FRSS-08	*T. orientale* AAUT17	27^{de}	39^{cdefgh}	43^{cd}	53^{b}	3
FRSS-14	*T. afroharzianum* AAUT30	14^{fgh}	29^{efgh}	40^{cd}	68^{ab}	2
FRSS-10	*T. afroharzianum* AAUT37	14^{fgh}	27^{gh}	43^{cd}	67^{ab}	2
FRSS-05	*T. afroharzianum* AAUT38	15^{fgh}	24^{h}	42^{cd}	64^{ab}	2
FRSS-16	*Trichoderma* sp. AAUT40	18^{efg}	30^{efgh}	42^{cd}	64^{ab}	2
FRSS-09	*T. tomentosum* AAUT44	19^{efg}	26^{gh}	42^{cd}	74^{ab}	2
FRSS-12	*T. afroharzianum* AAUT50	7.0^{h}	28^{gh}	43^{cd}	64^{ab}	2
FRSS-11	*T. afroharzianum* AAUT45	19^{efg}	36^{defgh}	47^{cd}	81^{a}	1
	CV	0.14	0.18	0.11	0.22	-

FRSS"- stands for faba bean rhizosphere soil samples with corresponding number. Mean values of three replications within the same columns labeled with same letter (s) are not significantly different ($p>0.05$) by Tukey HSD analysis of One-Way ANOVA. Numbers refer to antagonism reactions of *Trichoderma* isolates with *B. fabae* based on the scale of Bell et al. (1982) after 7 days of dual growth. CV-indicates the coefficient of variation among means in the same column.

The MGI over control was increased from 3rd-9th days ranging from 13%-64% (Figure 3.1) in sealed plate method. The most effective strain was *T. afroharzianum* AAUT19 that showed the highest activity upon all the incubation days, followed by *T. harzianum* AAUT14 and *T. harzianum* AAUT8.

All the strains reached the maximum inhibition of 50-60% upon the longest day of incubation, except in *T. tomentosum* AAUT4 and *T. harzianum* AAUT6. It is interesting to note that these strains, together with *T. afroharzianum* AAUT45, were the most effective antagonistic properties in a dual culture (PIRG>80%) and yet were not effective in this test. On the contrary, *T. afroharzianum* AAUT19 and *T. afroharzianum* AAUT35 that displayed relatively lower activity than the best performers in the dual assay, showed better activity in the MGI assay. The difference between the two assays, might be related to the efficiency of volatile metabolites produced by the isolates. In general, the highest %MGI was shown by *T. harzianum* AAUT14 having no significant variation ($p>0.05$) with *T. harzianum* AAUT8 and *T. afroharzianum* AAUT19 both having 63% of MGI after 3 (62%) and 6 (64%) days of incubation. However, there was variation ($p>0.05$) among the study isolates that might be caused by the production of different volatiles at different stages by the *Trichoderma* strains.

Trichoderma harzianum showed an inhibition of 47% against *Botrytis* isolates on 7 days of incubation (Bendahmane *et al*., 2012***)*** and the present study also indicated 30-62% of MGI by different *T. harzianum* strains. On the other side, the *T. harzianum* strains obtained from faba bean leaves exhibited 56-72% of MGI after 7 days of incubation as reported by Barakat et al. (2014) and Saber et al. (2009) found 50% mycelial growth inhibition of *B. fabae* by *T. harzianum* tag7 on the 6th days of incubation. However, the dual culture assay indicated better inhibition than sealed plate method to screen *Trichoderma* spp. after 9 days of incubation, whereby maximum of 64% and 88% MGI was displayed by *T. harzianum* AAUT14 on the same days of incubation in the volatile and volatile metabolites assays, respectively. The non-volatile assay conducted by dual culture method contains the non-diffusible substrates that might be released by the isolates into the medium and the involvement of diffusible metabolites that can synergistically be contributed to better antagonism than in the volatile (sealed plate method).

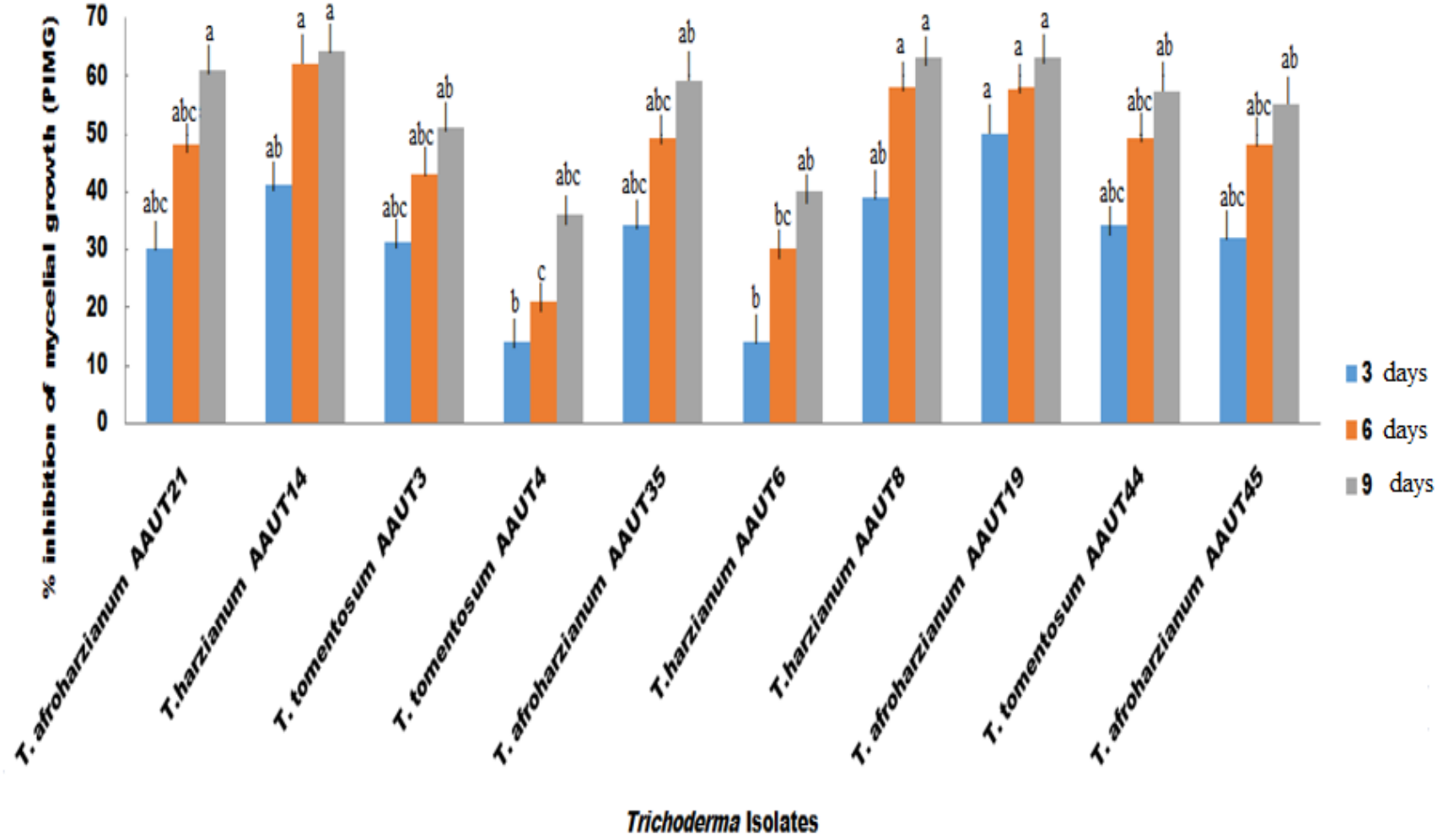


Figure 3.1: The effects of *Trichoderma* spp. on the mycelial growth of *Botrytis fabae* through sealed plate method. The same letter (s) on the graph represent no significance difference ($p>0.05$) on 3rd, 6th and 9th days of incubation by Tukey HSD analysis of One-Way ANOVA.

In the detached leaf assay, all the *Trichoderma* isolates illustrated the least level of *B. fabae* infection (<2.5 scale) in both faba bean varieties compared to their respective controls. *Trichoderma harzianum* AAUT14, *T. tomentosum* AAUT44 and *T. afroharzianum* AAUT45 allowed the least level *B. fabae* leaf infection but maximum leaf infection was seen in the controls that treated with *B. fabae* only (Table 3.4). In Ashebeka variety, treated by *T. harzianum* AAUT14, *T. tomentosum* AAUT44 and *T. afroharzianum* AAUT19, *B. fabae* displayed flecked lesion (1-1.3 mean growth of *B. fabae*). In addition, *B. fabae* showed flecked lesion (0.6 mean growth of *B. fabae*) in Hachalu variety treated with *T. harzianum* AAUT14 and 1.3 mean growth of *B. fabae* treated with *T. tomentosum* AAUT3 after 5 days of inoculation.

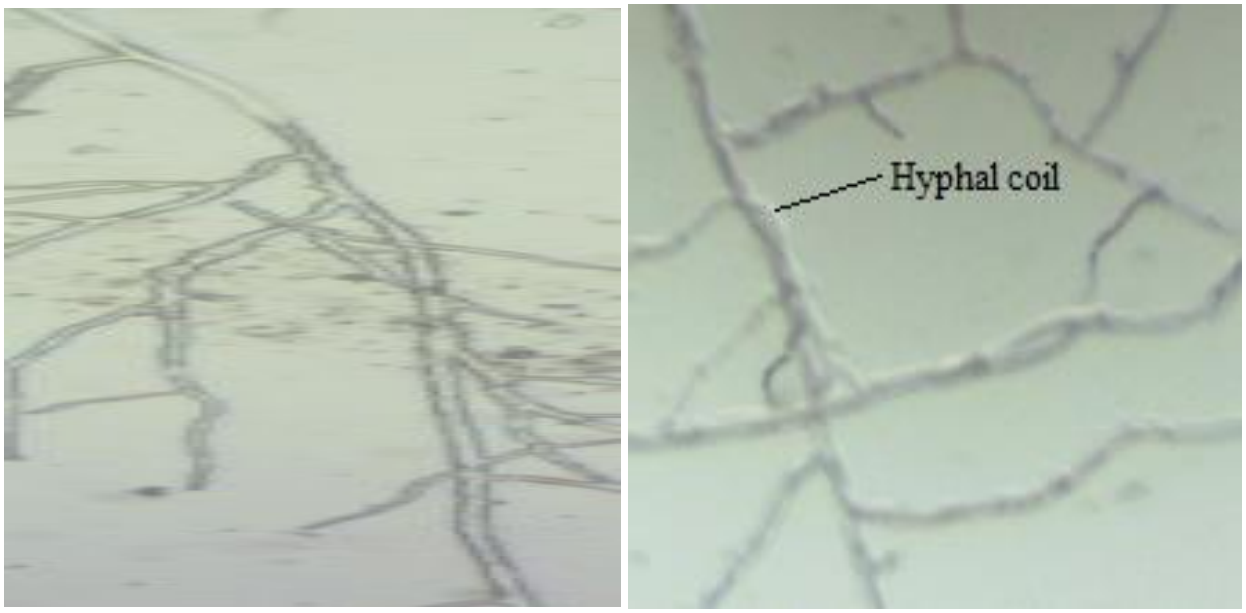
All the isolates were significantly different ($p<0.05$) compared to their respective faba bean variety control in the protection of *B. fabae* infection and its symptom development on the leaf samples of faba beans. This indicates the potential of the isolates in prolonging the incubation period of leaf samples more than 5 days. The same result was reported by Ermias Teshome et al. (2013) using different *Trichoderma* spp. to increase the incubation period of different faba bean leaf samples inoculated with *B. fabae* better than the controls. In addition, the reduced development of chocolate spot symptoms on different faba beans genotypes (Samuel Sahile *et al*., 2011).

Table 3.4: The effects of *Trichoderma* species on the developments of chocolate spot symptoms caused by *Botrytis fabae* on two faba bean varieties (Ashebeka and Hachalu) using detached leaf assay after 5 days of inoculation

Treatment	Mean growth of *B. fabae* (virulence scale 0-3)	
	Ashebeka	**Hachalu**
T. afroharzianum AAUT21	1.67[ab]	2.00 [ab]
T .harzianum AAUT14	1.00 [b]	0.67[b]
T. tomentosum AAUT3	2.00[ab]	1.33 [ab]
T. tomentosum AAUT4	1.67 [ab]	1.67[ab]
T. afroharzianum AAUT35	2.00 [ab]	1.67[ab]
T. harzianum AAUT6	1.67 [ab]	2.33[a]
T. harzianum AAUT8	1.67 [ab]	1.67[ab]
T. afroharzianum AAUT19	1.33 [ab]	2.00 [ab]
T. tomentosum AAUT44	1.00 [b]	1.67[ab]
T. afroharzianum AAUT45	1.67 [ab]	2.00 [ab]
Control	3.00 [a]	2.67[a]
CV	0.35	0.36

Mean values in the same column labeled with the same letter (s) as superscript are not significantly different ($p>0.05$) by Tukey HSD analysis of One-Way ANOVA. CV-indicates the coefficient of variation among means in the same column.

It was noted that the hyphae of all the *Trichoderma* grew on *B. fabae* and their hyphae formed hyphal coiling, indicating the mycoparasitic behavior of the isolates as represented by *T. harzianum* AAUT14 (Figure 3.2). Many studies have reported that most isolates of the genus *Trichoderma* to act as mycoparasites of many economically important faba bean fungal pathogens. For example, *Trichoderma harzianum* Rifai (Soliman *et al*., 2016), and *Trichoderma reesei* (Magdy *et al*., 2008) mycoparasitized *B. fabae* using slide culture studies. Therefore, in this study, the inhibitory activity of *Trichoderma* spp. in dual culture method is based on mycoparasitism that can cause hyphal lysis of *B. fabae*.



(a) (b)

Figure 3.2: Mycoparasitism of *T. harzianum* AAUT4 on *Botrytis fabae* (control (a), growth on *Botrytis fabae* and hyphal coiling (b)).

The data showed that the *Trichoderma* strains solubilized TCP, produced IAA and ammonia (Table 3.5). Thus, 47%, 63%, and 95% of the strains solubilized inorganic calcium phosphate, produced IAA and ammonia, respectively. The strains belonging to *T. harzianum* AAUT14, *T. tomentosum* AAUT4, *T. harzianum* AAUT6, *T. harzianum* AAUT8, *T. orientale* AAUT17, *T. afroharzianum* AAUT37 and *T. afroharzianum* AAUT38 produced the tested plant growth-promoting properties in this study.

Indole-3-acetic acid production was varied considerably among the isolates. The isolates produced IAA that ranged from 1.0-4.17 µg mL^{-1} indicating that *Trichoderma* spp. obtained from faba bean rhizosphere can act as growth promoter of plants through synthesizing beneficial phytohormones. Maximum IAA was produced by *T. harzianum* AAUT6 (4.17 µg mL^{-1}) followed by *T. afroharzianum* AAUT30 (3.34 µg mL^{-1}) and *T. harzianum* AAUT14 (3.16 µg mL^{-1}). The better quantity of IAA was produced by the isolates that showed inhibitory activity against *B. fabae*. Kumar et al. (2017) reported IAA producing *T. viride* VKF3 exhibited a maximum inhibition (82%) against *F. oxysporum*.

Similarly, 95% of the isolates produced ammonia. Prasad et al. (2017) have found as 95.5 and 64% of *Trichoderma* strains obtained from the rhizosphere region of tomato are the producers of ammonia and IAA, respectively. However, Mohiddin et al. (2017) have revealed 65% of the *Trichoderma* strains obtained from the rhizosphere of chili fields and Kitchen garden were positive for IAA, which is different from the present study. The difference might be associated with the source and or the strains potentials of producing IAA.

The production of ammonia may have contributed to the antagonistic property displayed by the *Trichoderma* strains against the studied pathogen by acting as volatile metabolite in this study. The production of ammonia by different *T. harzianum* strains has been widely documented as means to culminate the pathogens as a result of its cellular toxicity (Rawat and Tewari, 2011). Although, *Trichoderma* sp. AAUT10, showed various level of antagonism in the non-volatile (dual culture) assay, but the isolate failed to produce IAA, ammonia and solubilize the inorganic phosphate (TCP). This may show its sole antagonistic role in the proximity of the test pathogens through different mechanisms that are not determined in this study that may include the synthesis of lytic enzymes and siderophores. In general, the antagonistic *Trichoderma* isolates displayed multiple plant growth-promoting properties (MPGPP).

Table 3.5: Plant growth-promoting properties of *Trichoderma* isolates from faba bean (*Vicia faba* L) Rhizosphere grown in Arsi and Bale Zones

Identified species	IAA (µg mL^{-1})	Ammonia	Phosphate solubilization	NPGPP
Control	0.00^{g}	-	-	-
T. afroharzianum AAUT21	2.36^{cd}	+	-	2
T.harzianum AAUT14	3.16^{bc}	+	+	3
T. tomentosum AAUT3	-	+	-	1
T. tomentosum AAUT4	1.0^{ef}	+	+	3
T. afroharzianum AAUT35	1.70^{de}	+	-	2
T.harzianum AAUT6	4.17^{a}	+	+	3
T.harzianum AAUT8	1.03^{ef}	+	+	3
T. afroharzianum AAUT19	1.06^{ef}	+	-	2
Trichoderma sp. AAUT10	-	-	-	0
T. tomentosum AAUT16	-	+	-	1
T. orientale AAUT17	1.03^{ef}	+	+	3
T. afroharzianum AAUT30	3.34^{ab}	+	-	2
T. afroharzianum AAUT37	-	+	+	2
T. afroharzianum AAUT38	2.67^{bc}	+	+	3
Trichoderma sp. AAUT39	1.21^{e}	+	-	2
Trichoderma sp. AAUT40	-	+	+	2
T. tomentosum AAUT44	-	+	-	1
T. afroharzianum AAUT50	-	+	+	2
T. afroharzianum AAUT45	2.54^{bcd}	+	-	2
Total (%)	63	95	47	95

NPGPP-Number of plant growth promoting properties (0-3 in number). Mean values in the same column labeled with the same letter (s) as superscript are not significantly different ($p>0.05$) by Tukey HSD analysis of One-Way ANOVA.

The phosphate solubilizing strains were further tested for their ability to solubilize inorganic phosphate (TCP) in a liquid culture medium quantitatively (Table 3.6). Different *Trichoderma* strains showed variations in the concentration of solubilized phosphate that ranged from 135-575 µg mL^{-1} upon 3-6 days of incubation, exhibiting significant difference ($p<0.05$) with the control. Tricalcium phosphate solubilization increased from 3^{rd}-6^{th} days and reduced after the 6 days. *T. harzianum* AAUT6 released 338 µg mL^{-1} of solubilized phosphate on the 3^{rd} days and followed by *T. harzianum* AAUT14 (327 µg mL^{-1}) and *T. tomentosum* AAUT4 (326 µg mL^{-1}). The quantity of released phosphate increased from 180-575 µg mL^{-1} after 3^{rd} days and solubilization reduction was observed after the 6 days of incubation in all the isolates, though, maximum phosphate was released on the 6 days of inoculation by *T. afroharzianum* AAUT38 (575 µg mL^{-1}). Compared to the respective controls, an increase of 2-7, 4-11 and 1.75-6.89 fold was displayed on the 3, 6 and 9 days of inoculation, respectively.

This result was much higher than Kapri and Tewari (2010) who pointed out 392.96 µg mL^{-1} of solubilized phosphate obtained from *Trichoderma* DRT-1 upon 6 days of incubation. However, Bader et al. (2020) have reported a maximum of 288.18 µg mL^{-1} solubilized TCP by *Trichoderma* FCCT 363-2 strains on 6 days of inoculation, which is different from the peak concentration noted in the present study (509 µg mL^{-1}) by *T. harzianum* AAUT14 strain.

Table 3.6: Phosphate solubilization efficiency of the different *Trichoderma* isolates from faba bean (*Vicia faba* L.) rhizosphere; the amount of phosphorus released (µg mL^{-1}) (NBRIP liquid) (from inorganic calcium phosphate based upon days of incubation (3-9) (for isolates with PVK"+") and change in the initial pH of medium (pH_o=7)

Identified species	TCP solubilization 5gL^{-1} NBRIP (liquid) (µg mL^{-1})			pH change (3-9 days)		
	3	**6**	**9**	**3**	**6**	**9**
Control	45 ^{f}	47 ^{h}	49 ^{f}	6.4 ^{a}	6.6^{a}	6.5 ^{a}
T.harzianum AAUT14	327 ^{a}	509^{b}	351 ^{c}	5.5 ^{bcd}	4.3^{bc}	5.0^{bcd}
T. tomentosum AAUT4	326 ^{a}	428 ^{c}	387^{b}	5.8 ^{b}	4.1^{bcd}	5.2^{bc}
T.harzianum AAUT6	338^{a}	386^{d}	239 ^{d}	5.0^{de}	4.3^{bc}	5.4^{b}
T.harzianum AAUT8	135^{e}	216 ^{f}	157^{e}	5.6 ^{bc}	4.5^{b}	5.3^{bc}
T. orientale AAUT17	236^{c}	294 ^{e}	231^{d}	5.1^{cde}	4.3^{bc}	4.7^{cd}
T. afroharzianum AAUT37	242^{c}	417^{c}	357 ^{c}	5.1^{cde}	4.1^{bcd}	4.5^{c}
T. afroharzianum AAUT38	285^{b}	575^{a}	417 ^{a}	5.0^{de}	4.5^{b}	4.9^{c}
T. afroharzianum AAUT50	143^{d}	180^{g}	135^{e}	4.7 ^{e}	4.2^{bcd}	4.5^{c}
CV	0.30	0.32	0.32	0.18	0.22	0.20

Mean values in the same column labeled with the same letter (s) as superscript are not significantly different ($p>0.05$) by Student-Newman-Keuls (S-N-K) analysis of One-Way ANOVA. CV- indicates the coefficient of variation among means in the same column.

The data also showed a steady decrease of 1-2.5 pH units from pH of 6.6-4.1 from 3-6 days of incubation. This may indicate that phosphate solubilization might be enhanced in acidic conditions as it was observed on the 6th days after inoculation with *T. afroharzianum* AAUT38 (575 µg mL^{-1}, pH=4.5), *T. harzianum* AAUT14 (509 µg mL^{-1}, pH=4.3) and *T. tomentosum* AAUT4 (428 µg mL^{-1}, pH=4.1). Although *T. tomentosum* AAUT4 displayed a pH of 4.1, the released phosphate was the lower than observed in the two isolates (*T. afroharzianum* AAUT38 and *T. harzianum* AAUT14) on the same days, which may indicate the involvement of other mechanisms for TCP solubilization. According to Ribas (2016), the potential of TCP solubilization in *Trichoderma* spp. is not only assisted by medium acidification, but also the production of alkaline phosphatases (ALP). The Pearson`s analysis indicated a negative correlation (Table 3.7) between pH and TCP solubilization (r=-0.612**). Asea et al. (1998) has also indicated a negative correlation between pH and the amount of solubilized phosphorus in liquid medium. However, after the 6th days of inoculation an increase in the pH was observed that might be caused by alkaline substances that are released by the strains into the medium.

Table 3.7: The Pearson`s correlation analysis between Phosphate solubilization and the change of pH in the medium

Correlations

		phosphate solubilized	pH change
phosphate solubilized	Pearson Correlation	1	-.612**
	Sig. (2-tailed)		.001
	Sum of Squares and Cross-products	500422.000	-1574.900
	Covariance	19247.000	-60.573
	N	27	27
pH. change	Pearson Correlation	-.612**	1
	Sig. (2-tailed)	.001	
	Sum of Squares and Cross-products	-1574.900	13.250
	Covariance	-60.573	.510
	N	27	27

**. Correlation is significant at the 0.01 level (2-tailed).

3.8 Conclusion and Recommendations

This study pointed out the antagonistic potential of different *Trichoderma* strains on *Botrytis fabae* with producing plant growth-promoting traits. *T. harzianum* AAUT14 showed the best antagonistic feature against *B. fabae* along with different plant growth-promoting properties. Thus, *T. harzianum* AAUT14 strain can be a candidate to be further evaluated as biofungicide of *B. fabae* either under greenhouse and or field study in combination with elite bacterial isolates or alone.

Based on the findings of this study, it could be recommended that further studies should be conducted on the compatibility of *Trichoderma* spp. with fungicides to be utilized as an integrated disease management (IDM).

CHAPTER 4

The effects of Selected of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on the control of chocolate spot and faba bean (*Vicia faba* L.) performance under greenhouse conditions

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Abstract

Chocolate spot is a fungal disease caused by fungal pathogens *Botrytis fabae*/*cinerea*. The yield loss caused by the disease is reported to be 67.5% in Ethiopia. There are several methods employed to control the disease of which the application of biological control agent is an alternative to boost crops productivity. In this study, a completely randomized design was used to evaluate the antagonistic effects of *T. harzianum* AAUT14 (T2), *B. subtilis* AAUB95 (T3), *T. harzianum* AAUT14+*B. subtilis* AAUB95 (T4) in comparison to fungicides, MORE 720 WP (T5) and ORZEB 80 WP (T6) against chocolate spot (*B. fabae*) using two faba bean varieties (Ashebeka and Hachalu). The data were analyzed by SPSS version 24. On the 60 days of treatment, the bioagents reduced the disease incidence to 36-50% on Ashebeka variety compared to the control (T1), by which T4 displayed the highest reduction (50%) showing no significance difference ($p>0.05$) with T6. On the other hand, the bioagent received plants showed 34-39% reduction of disease incidence upon 80 days, where by T4 showed insignificant difference ($p>0.05$) with T6. However, reduction in the disease severity was pronounced upon 80 days ranging from 41-50% by the bioagents compared to the fungicides that showed 55% reduction. T4 inoculated plants showed the disease incidence reduction of 54% having no significance variation ($p>0.05$) with T5(56%). Nevertheless, T4 showed no significance difference ($p>0.05$) on reducing the disease severity with both fungicides (T5 and T6) upon 80 days on Hachalu variety. An AUDPC of 405.97% and 415.33% was displayed by T5 and T6, respectively in Ashebeka variety and 377.98% and 412.48% of AUDPC was illustrated by T5 and T6 in Hachalu variety, respectively with no

significance difference ($p>0.05$) from T4. On the other hand, the shoot dry weight displayed 56% in Ashebeka and 60% increase over T1 in Hachalu variety. In addition, the shoot nitrogen content increased from 40-82% in Ashebeka and 36-88% in Hachalu variety over T1. In the same way phosphorus content improved from 22-88% and 21-84% in Ashebeka and Hachalu varieties, respectively by the treatments, whereby the highest increment was achieved by the T4 received plants. Therefore, this study indicated that the mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 performed better than when separately applied. Thus, the combination can be further validated under field conditions.

Keywords: *Botrytis fabae*; Combined inoculation; Disease severity; Fungicides; *in vivo*

4.1 Introduction

Chocolate spot is a fungal disease caused by fungal pathogens *Botrytis fabae*/*cinerea*. The yield loss caused by the chocolate spot can reach as high as 50-100% in Australia, Egypt, England and China depending upon the climatic conditions (Abo-Hegazy *et al*., 2012). In Ethiopia, the yield losses of up to 67.5% was recorded in susceptible faba bean cultivars by chocolate spot disease (Samuel Sahile *et al.*, 2010). There are several methods employed to control the disease of which the application of chemicals is the most important approach in modern and large scale agricultural activity. However, this approach is not affordable to the low-input agriculture with few acres of land in developing countries.

This requires the use of alternative methods such as using biological control agents (BCAs) in an integrated disease management (IDM) system that can reduce the use of chemicals, environmental pollution and boost the productivity of crops. In this regard, microorganisms play an important role in serving as biocontrol inputs in IDM, of which *Trichoderma* and *Bacillus* are found to be an important candidate. In this regard, several BCAs are now emerging for separate use in disease control, or in combination with chemical pesticides. *Trichoderma* and *Bacillus* are two of the important groups of rhizosphere microorganisms that have profound effects on the growth, nutrition and health of plants (Choudhary *et al*., 2018).

Different studies have been conducted on the beneficial aspects of *Trichoderma* spp. in different plants. These fungi, as growth promoter has been demonstrated with yield increase of 84.9% in strawberries (Porras *et al*., 2007). The dual culture and volatile metabolites assays showed that *T. harzianum* suppressing the growth of *Fusarium solani* by 51.4 and 38.1%, respectively and tomato plants treated by the same strains showed 117.5 and 138.9% increase in plant height and dry weight, respectively and with the disease incidence reduction of 55.5% compared to the uninoculated controls (Bokhari and Perveen, 2012). *Trichoderma harzianum* that showed antagonistic activity against *F. oxysporum* and *R. solani* resulted in 65%, 70%, and 58% increase of shoot length, root length and root dry weight in chickpea, respectively, compared to the control (Yadav *et al*., 2011).

Similarly, *Bacillus* spp. are widely distributed in the soil and plant rhizosphere. Ajilogba et al. (2013) have studied the antagonistic effects of *Bacillus* species as biocontrol tools of tomato fusarium wilt under *in vitro* and *in vivo* study. They showed that *B. amyloliquefaciens* displayed 95.2% growth inhibition on *F. solani*, while *B. cereus* showed growth inhibition of 55.7%. Mangalanayaki and Durga (2013) have reported that *B. pumilus* and *B. subtilis* controlled the growth of *F. solani* by 95.20% and 30.50%, respectively. *Bacillus* spp. also displayed 23-64% inhibition (Samuel Sahile *et al*., 2009), Ahmed (2015) and El-Banoby et al. (2013) have reported the inhibitory activity of *Bacillus subtilis* on the mycelial growth of *B. fabae*, by 62.6% and 67.03%, respectively.

The success of *Trichoderma* and *Bacillus* spp. in the rhizosphere region is due to their high reproductive capacity, survival under unfavorable conditions, nutrient utilization efficiency and their strong aggressiveness against plant pathogenic fungi (Harman, 2006). These properties are associated with the ability to produce peptide antibiotics and contribute to the utilization of these isolates to manage several plant diseases. Because of this, these microbes have great importance in the modern agricultural activity due to their predominance in the rhizosphere, potential of phytopathogens control and growth promotion in plants (Sharma *et al*., 2013). Thus, the co-inoculation of microorganisms is becoming an important component in the management of plant disease in the modern agricultural activity. Ali and Nadarajah (2013) have showed that the dual inoculation of seeds with a mixture of *Trichoderma* spp. and *B. subtilis* showed the highest ability to suppress rice sheath blight caused by *R. solani* and enhanced the productivity.

Chocolate spot is one of the fungal diseases expanding in different parts of the world (Hebblethwaite, 1983). In Ethiopia, the disease is widely expanding to different parts of the country compared to other fungal diseases such as rust and root rot in faba beans. Attempt is made to control the disease using different fungicides (Kabbabeh *et al*., 2009). However, the rise in the cost of these fungicides from time to time and resistance of *B. fabae* to various fungicides has been detected as a problem to manage the disease caused by this pathogen (Hassan *et al*., 2006). Therefore, it is essential to identify sustainable approaches to manage this disease using biofungicides as BCAs.

To this effect, several studies have been undertaken to see the single and dual inoculation of *Bacillus* and *Trichoderma* spp. to control the disease under laboratory conditions (Barakat *et al.* 2014; El-Banoby *et al*., 2013; Mbazia *et al*., 2016; Samuel Sahile *et al.*, 20011; Teshome *et al.* 2013)

However, there is a dearth of information on combined inoculation of *Bacillus* spp. and *Trichoderma* spp. as biofungicides is lacking against this pathogen under greenhouse condition. Therefore, this study was initiated to evaluate the effects of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 against chocolate spot (*B. fabae*) and to assess their growth-promoting properties of faba bean under greenhouse conditions.

4.2 MATERIALS and METHODS

4.2.1 Sources of Inoculants, Host varieties, the Pathogen and Fungicides

Trichoderma harzianum AAUT14 and *B. subtilis* AAUB95 (antagonistic agents), *B. fabae* (phytopathogen) were obtained from our previous *in vitro* studies. The rhizobial strain, *Rhizobium leguminosarum bv. viciae* (FB-1035) and the two faba bean (Ashebeka and Hachalu) varieties were obtained from Holleta Agricultural Research Center (HARC). The fungicides, viz, MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80WP (Mancozeb) were bought from the local market and applied to faba bean according to the manufacture's instruction given on the packs.

4.2.2 Compatibility Assay of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 Under *in vitro* Conditions

the compatibility of *Trichoderma* and bacterial spp. an in vitro dual culture assay was done on potato dextrose agar (PDA) medium modified with 10% sucrose according to Yobo (2005). The bacterial strains grown on nutrient agar for 24 hrs. were taken and spot inoculated using sterile inoculating loop at the four corner of Petri plate and incubated at 28±2°C for 48hrs. A mycelial agar cut having 5mm diameter from *Trichoderma* spp. was then centrally inoculated on Petri plates that were pre-inoculated with rhizosphere bacteria. The plates were then incubated for 120 hrs. at the same temperature. The compatibility of the *Trichoderma* spp. with bacterial isolates was checked by the mycelial overgrowth on the bacterial strains with creating no inhibition zone through visual observation. In this assay the bacterial species that showed the presence of inhibition zone to *Trichoderma* spp. were considered incompatible and excluded from their incorporation to the study.

4.2.3 Greenhouse Experiment Treatments and Experimental Design

The experiment was done to evaluate the potential of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 as single and dual inoculants against chocolate spot disease of faba beans. Pots

measuring 9 cm in width and 18 cm in height were utilized for faba bean growing in the greenhouse.

Six treatments and three replications per treatments and each treatment was planted with four seeds of faba bean in a completely randomized design (CRD). Two faba bean (Ashebeka and Hachalu) varieties were utilized for this experiment. The fungicides were applied according to the instruction given on the packs as foliar spray following the onset of chocolate spot symptom. Diammonium phosphate (DAP, 46% P_2O_5 and 20% P) was supplemented to all the treatments at 100 kg ha^{-1} at the time of planting (EIAR, 2018). The experiment included the following treatments in both varieties.

T1-Control (*B. fabae* only)
T2- *T. harzianum* AAUT14+*B. fabae*
T3- *B. subtilis* AAUB95+*B. fabae*
T4- *T. harzianum* AAUT14+*B. subtilis* AAUB95+*B. fabae*
T5- MORE 720 WP+*B. fabae*
T6- ORZEB 80 WP+*B.fabae*

4.2.4 Preparation of *Botrytis fabae* and Soil Infection

Botrytis fabae was grown on potato dextrose agar (PDA) at 25±2°C for 10-12 days and slurry of culture was made by adding sterilized water to the plate surface having the mycelia of *B. fabae*. The mycelia were scraped using sterile forceps and the suspension was filtered through sterilized gauze, the spore concentration was adjusted to 4.5×10^5 spore mL^{-1} using haemocytometer (Hanounik and Hasanain,1986). Soil and sand was mixed in the ratio of 3: 1 and sterilized at 121°C for 30 min. Pots containing the sterilized soil and sand mix (3:1 ratio) was infected with the adjusted spore concentration of *B. fabae* at the rate of 48 mL/pot 7 days prior to planting.

4.2.5 Inocula Preparation, Seed Coating and Foliar spray

The inoculum of *T. harzianum* AAUT14 was prepared according to Navaneetha et al. (2015). A 5 mm mycelial disc was inoculated on fresh PDA and incubated at 25±2°C for five days. After

incubation, 10 mL sterile water was added to the plates, scrapped slightly using the suspension, 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 (Mohammed *et al.*, 1994). *Bacillus subtilis* AAUB95 was cultured in 100 mL flasks containing 40 mL nutrient broth. The flasks were incubated at room temperature on shaker at 130 rpm for 48 hrs. and $1x10^9$CFU mL^{-1} of cells were utilized for the study (Sharga, 1997). The CFU/mL of the strain was checked by plate count method. Faba bean seeds, were washed with tap water, surface sterilized by 3% sodium hypochlorite for 1 min and rinsed in distilled-sterilized water thoroughly. Seeds were air dried over the sterilized filter paper under fume hood and checked for germination on Petri plate.

Faba bean seeds that found germinating were treated with the suspensions of *T. harzianum* AAUT14 at 2.5×10^5 spore mL^{-1} (Mohammed *et al.*, 1994), *B. subtilis* AAUB95 at $1x10^9$CFU mL^{-1} (Sharga, 1997) and 1mL of *R. leguminosarum bv, viciae* (FB-1035) containing $1x10^9$ CFU mL^{-1} (Benidire *et al*., 2017) as seed coating using 10% carboxyl methyl cellulose (CMC) and aseptically planted into pre-prepared pots for 85 days. In addition to seed coating, foliar spraying of the developed plants with the spore suspension of the same bioagents (*T. harzianum* AAUT14 and *B. subtilis* AAUB95) were done two times at the 35^{th} and 55^{th} days after sowing with 3 mL/pot (Saber *et al*., 2009; Sharga, 1997).

4.2.6 Disease Assessment and Data collection

After 60 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 stages of symptoms development. Disease severity was expressed as percent of affected leaf based on symptoms appeared according to Gullino et al. (2017), 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 of symptoms. An area under disease progress curve (AUDPC) in percentage of development stage unit (%DSU) was calculated according to Hanounik and Hasanain (1986).

%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, DS-disease severity, DI-disease incidence, 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 and AUDPC- area under disease progress curve. 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 (%DSU).

4.2.7 Analysis of shoot nitrogen and phosphorus contents

4.2.7.1 Phosphorus analysis

The phosphorus analysis was done by vanadium phosphomolybdate wet digestion method as described by Motsara and Roy (2008). One gram (1 g) of plant sample was digested with the addition of 10 mL acid mixtures HNO_3, H_2SO_4 and $HClO_4$ in the ratio of 9:4:1), the volume was made up to 100 mL. A 3.5 mL of the digest was transferred into a 50 mL volumetric flask and 10 mL of vanadomolybdate was added, the volume was made to 50 mL with distilled water, shaked thoroughly and left for 30 min. The development of yellow colour was then read at 880 nm spectrophotometrically (6405UV/Vis., Jenway, England) and the concentration was calculated based on the equation obtained from the standard graph sketched based on known concentration of potassium di-hydrogen orthophosphate, KH_2PO_4 (µg mL^{-1}) and expressed in percentage (%).

4.2.7.2 Total Nitrogen Analysis

Total nitrogen in leaf samples was determined by Kjeldahl method. One gram (1g) leaf samples were digested with 10 mL of concentrated H_2SO_4 and kept for 1 hr. A 1g of selenium was added, the mix was digested at 375°C for 2 hrs. The digest was cooled and 150 mL distilled water and 45

mL of 45% NaOH was added. The mixture was then distilled with 30 mL of 2% boric acid and the percentage of nitrogen in the samples was calculated by using the following formula (Motsara and Roy, 2008).

$$\%N = \frac{(V-B) \times N \times Equi.wt.N \times 100}{1000 \times wt.(g)}$$

Where, V-volume of the titrant, N-normality of the titrant, Wt.- weight of the sample, B- blank and Equiv. wt. of N = 14

4.2.8 Data Analysis

The statistical analysis was performed by SPSS version 24. The interaction among disease, hosts and days of disease assessment was conducted by Three-Way ANOVA and means were compared by Tukey HSD analysis of Two-Way ANOVA. All the values were considered statistically significant at $p<0.05$.

4.3 Results and Discussion

The compatibility assay between *T. harzianum* AAUT14 and *B. subtilis* AAUB95 showed no inhibition to each other with the absence of any inhibition zone (Figure 4.1). Similar observations have been reported by Zaim et al. (2018) between the *B. subtilis* Bs1 and *T. harzianum* T5 under *in vitro* conditions. It should be pointed out that the use of mixture or combinations of different strains can be practiced provided that no member of the mixture is inhibitory to another or interferes excessively with other microorganisms existing in the rhizosphere (Whipps, 2001).

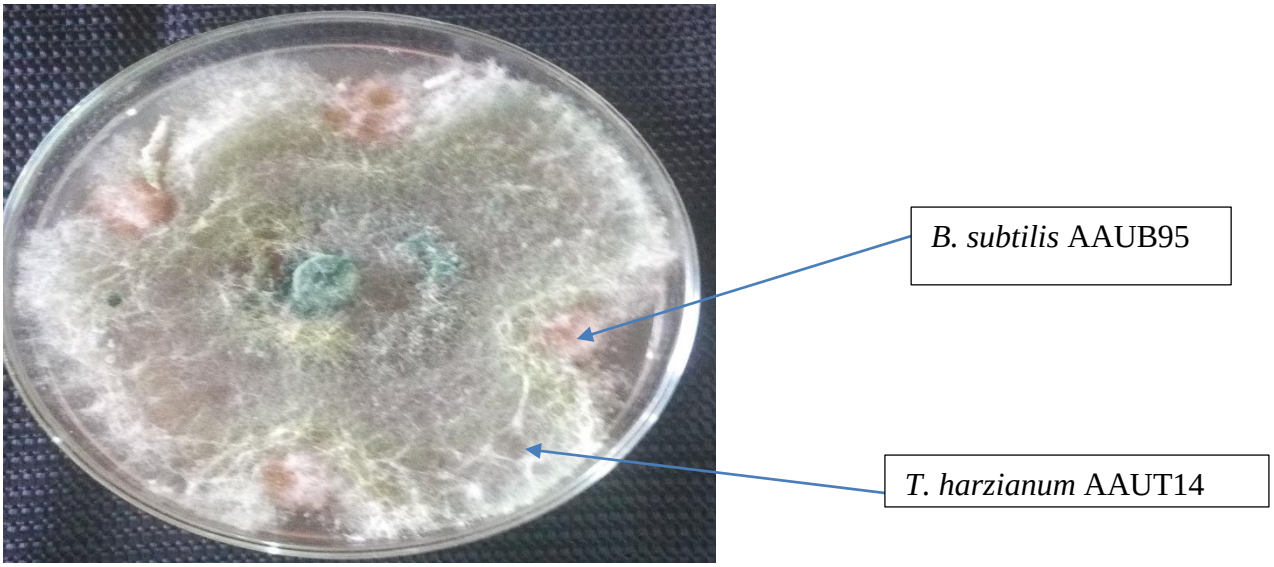

Figure 4.1. An *in vitro* compatibility assay between *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAU95 after five days of inoculation on potato dextrose agar amended with 10% of sucrose at 25 °C.

The microbial and fungicide treated plants showed significant difference in reducing the disease incidence and severity on the two faba bean varieties at different days of treatment (DoT). Upon 60 days of inoculation, the disease incidence was reduced on Ashebeka variety by 36-62% compared to the uninoculated and chemically untreated control plants, T1 (Table 4.1). The dual inoculation of the two antagonistic microbes, *T. harzianum* AAUT14+*B.subtilis* AAUB95 (T4) displayed the highest reduction of 50% in disease incidence. Although the effectiveness of the microbial treatment was the highest, the result was lower than the 62% reduction exerted by the

fungicide MORE 720WP (T5), and it was slightly higher than the 45% reduction effected by the other chemical fungicide**,** ORZEB 80WP (T6). The single inoculation of the two antagonists did not show significant difference ($p>0.05$) with one another and exhibited lower reduction compared to the other treatments.

Upon 80 days of treatment, the inoculated plants showed 34-51% reduction in disease incidence together with T6. All these treatments showed lower incidence than the T5 treated plants that displayed 51% disease incidence on the Ashebeka variety (Table 4.1). According to Abdel-Kader et al. (2012), the single application of *T. harzianum* reduced the incidence of powdery mildew to 13.3% in cucumber and 16.6-33.3% in pepper comparing with 50.0 and 40.0% in control treatment, respectively. In the same study, *B. subtilis* alone reduced the incidence of powdery mildew to 16.6% in cucumber and 20% in pepper comparing with the same control, which is lower than this study. The difference might be associated with studied pathogen and or the host plants.

Table 4.1: The effect of single or combined applications of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on faba bean (Ashebeka) variety chocolate spot (*Botrytis fabae*) disease incidence and severity using pot experiments

Treatments	Days after treatment								AUDPC (%DSU)
	60				80				
	%DI	%R	%DS	%R	%DI	%R	%DS	%R	
T1-Control (B.f only)	13.68^{a}	-	32.00^{a}	-	28.49^{a}	-	64.00^{a}	-	702.13^{a}
T2-*T. harzianum* AAUT14+B.f	8.62^{b}	37	26.55^{bc}	17	18.69^{bc}	34	34.07^{bc}	46	469.32^{bc}
T3-*B. subtilis* AAUB95+B.f	8.73^{b}	36	27.00^{bc}	16	18.35^{bc}	36	37.33^{b}	41	489.90^{b}
T4-*T. harzianum* AAUT14+*B.subtilis* AAUB95+B.f	6.80^{bc}	50	24.11^{c}	25	17.32^{bc}	39	31.33^{bc}	50	409.77^{c}
T5-MORE 720 WP+B.f	5.22^{c}	62	23.55^{c}	26	13.89^{c}	51	28.29^{c}	55	405.97^{c}
T6-ORZEB 80 WP+B.f	7.59^{bc}	45	28.22^{ab}	12	18.26^{bc}	36	28.15^{c}	55	415.33^{c}
CV	0.33	-	0.11	-	0.29	-	0.35	-	0.22

B.f- *Botrytis fabae*, DI-Disease incidence, DS-Disease severity, %R- Percentage of disease incidence and severity reduction over the control, AUDPC- Area under disease progress curve, CV-Coefficient of variation. Mean values of three replications within same columns labeled with same letter (s) as superscript are not significantly different ($p>0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

The effectiveness of the different treatments also showed variations in disease severity on the Ashebeka variety as presented in Table 4.1 Upon 60 days of treatment, the treatments showed a disease severity reduction of between 12% and 26% compared to the control plants. Interestingly, both T4 inoculated plants and T5 treated plants showed the highest reduction in disease severity to the T3 inoculated plants (16%) and the T6 treated plants (12%). The reduction in disease severity was much pronounced upon longer days of treatment (80 days) ranging from 41% up to 55% compared to the disease incidence which did not show difference as a function of prolonged days of treatment. The T4 treated plants showed 50% reduction in disease severity compared to 55% reduction of the chemically treated (both T5 and T6) plants of the Ashebeka variety. Ermias Teshome et al. (2013) have reported that individually applied strains of *T. harzianum*, *T. gamsi* and *T. polysporum* lowered disease severity of chocolate spot to 42.22-51.11%, which is different from the present study.

The difference might be caused by the soil nutrients composition that were utilized for growing faba beans and or the inoculum size utilized along the ways of application. In this study, the authors used a mixture of sand, manure and compost in 1:2:3 ratios as a nutrient for faba bean growing in pots that can enhance the performance of the bioagents, whereas in the present study the mixture of sand and soil (3:1) were utilized. Behairy et al. (2014) found that *B. subtilis* and *T. harzianum* single inoculation reduced the diseases severity of alternaria leaf spot by 89% and 79%, respectively in faba beans, which is greater than the present study. The variation between this and their study might be associated with the difference in the studied pathogens, *Alternaria alternata* and *B. fabae* or the methods by which the biological control agents and the pathogens were applied or the inoculum size utilized. In their study, *Alternaria alternata* was inoculated on 45 days' plants of faba beans with the spore suspension of $1x10^6$ spore/mL on the second days *B. subtilis* and *T. harzianum* application.

The microbial and chemical treatments also showed similar trends in disease incidence and disease severity on the Hachalu variety (Table 4.2). The microbial inoculants reduced disease incidence in the range of 3.8% and 34% upon 60 days of treatment and 35% and 56% in 80 days of treatment. The T4 inoculated plants showed the highest reduction in disease incidence especially at the latter days (80 days) of treatment (54%) compared to T5 (56%) and T6 treated plants (53%). In a study

conducted by Ermias Teshome et al. (2013), *T. gamsi* gave the chocolate spot disease incidence reduction of 66.67%, followed by *T. harzianum* (61.67%) and *T. polysporum* (59%) compared to the control, 100%, which seems as single application of *Trichoderma* species could perform equal to the co-inoculation of *Trichoderma* with *Bacillus* spp. However, an equal amount of inoculum size was utilized for both the pathogen (*B. fabae*) and the *Trichoderma* spp. ($2.5x10^5$ spores/mL) in their study, which is lower than the inoculum size utilized for *B. fabae* during soil infestation (4.5×10^5 spore mL^{-1}) in the present study. This could be the cause for the better performance of the *Trichoderma* strains in their study. Furthermore, the percent reduction of disease showed an increase as a function of planting days following the occurrence of chocolate spot symptoms in both varieties. This might be due to the induction of systemic resistance (ISR) by the bioagents applied and the systemic acquired resistance (SAR) by the hosts (faba beans). That can result in the prevention of the symptoms and or minimize the number of lesions displayed by the pathogen.

With regard to disease severity, there was a drastic reduction in inhibition through T5 (54%) compared to the T4 (47%). Interestingly, the single inoculated plants with T2 showed effective inhibition (35%) that followed by T4 and T5 treated plants (49%). In all cases, T3 inoculated plants performed less than all the other treatments. This might be correlated with the less potency of the strain to control the studied pathogen when used alone.

Table 4.2: The effect of single and combined applications of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on faba bean (Ashebeka) chocolate spot (*Botrytis fabae*) disease incidence and severity using pot experiments

Treatments	Days after treatment								AUDPC (%DSU)
	60				80				
	%DI	%R	%DS	%R	%DI	%R	%DS	%R	
T1-Control (B.f only)	10.47 ^{a}	-	31.80^{a}	-	30.34^{a}	-	59.22^{a}	-	708.60^{a}
T2-*T. harzianum* AAUT14+B.f	7.56 ^{ab}	28	26.55^{bc}	17	17.32^{bc}	43	38.28^{bc}	35	496.57^{b}
T3-*B. subtilis* AAUB95+B.f	10.07 ^{ab}	3.8	27.00^{b}	15	19.68^{b}	35	47.34^{b}	20	554.65^{b}
T4-*T. harzianum* AAUT14+*B.subtilis* AAUB95+B.f	7.04^{b}	33	24.11^{bc}	24	13.88^{c}	54	31.52^{cd}	47	411.92^{c}
T5-MORE 720 WP+B.f	6.96^{b}	34	23.55^{c}	26	13.35^{c}	56	27.12^{c}	54	377.98^{c}
T6-ORZEB 80 WP+B.f	9.15 ^{ab}	13	25.23^{bc}	21	14.26^{bc}	53	30.01^{cd}	49	412.48^{c}
CV	0.24	-	0.11	-	0.36	-	0.31	-	0.24

B.f- *Botrytis fabae*, DI-Disease incidence, DS-Disease severity, %R- Percentage of disease incidence and severity reduction over the control, AUDPC- Area under disease progress curve, CV-Coefficient of variation. Mean values of three replications within same columns labeled with same letter (s) as superscript are not significantly different ($p>0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

In this study, the application of T2 and T3 alone reduced the disease severity in both varieties showing no significant variation to each other ($p>0.05$). This may imply the reason for better performance when the two strains were combined together as seen from the data of T4 in the present study. However, both the disease incidence, and disease severity percent of reduction showed an increase as a function of planting days in both varieties. This indicates the potency of the treatments to reduce the symptom of chocolate spot occurrence and its associated injury (leaf lesion) on the studied host (faba beans). On the other way the interaction analysis among the chocolate spot disease (disease severity and disease incidence), host varieties (Ashebeka and Hachalu) and days of disease assessment was found significantly ($p<0.05$) different (Table 4.3).

Table 4.3. An interaction analysis among the chocolate spot disease level (disease severity and disease incidence), hosts and days of disease assessment by Three- Way ANOVA

Tests of Between-Subjects Effects

Dependent Variable: chocolate spot disease

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	7649.624[a]	7	1092.803	31.864	.000	.621
Intercept	90626.085	1	90626.085	2642.501	.000	.951
days	1361.610	1	1361.610	39.702	.000	.226
disease	5682.898	1	5682.898	165.704	.000	.549
hosts	.570	1	.570	.017	.898	.000
days * disease * hosts	168.654	1	168.654	4.918	.028	.035
days * disease	279.113	1	279.113	8.138	.005	.056
days * hosts	29.052	1	29.052	.847	.359	.006
disease * hosts	127.728	1	127.728	3.724	.056	.027
Error	4664.198	136	34.296			
Total	102939.907	144				
Corrected Total	12313.822	143				

a. R Squared = .621 (Adjusted R Squared = .602)

In addition, AUDPC that ranged from 405.97-702.13% and 411.92 -708.6% was shown by the different treatments on Ashebeka and Hachalu variety, respectively. The maximum AUDPC observed was 702.13% by T1 in Ashebeka and 708.6% in Hachalu variety. Dagne Kora et al. (2017) have demonstrated that fungicides unsprayed faba bean, with 1817%,1476%, 1467% and 1716% of AUDPC on Sinana local, Shallo, Mosissa and Walki varieties, respectively. Their finding is different from this study which might be caused by the difference in the faba bean varieties used in the study and or the conditions of the study. Nevertheless, in this study, T4 demonstrated 409.77% and 411.92% of AUDPC in Ashebeka and Hachalu variety, respectively. Thus, this may indicate as the chocolate spot symptom development was significantly influenced by the treatments. Besides, T4 revealed no variation ($p>0.05$) with fungicides utilized, which may indicate the efficiency of dual inoculation than applying either T2 and/ or T3. An AUDPC of 405.97% and 415.33% was displayed by T5 and T6 in Ashebeka variety, respectively. In the same manner, 377.98% and 412.48% of AUDPC was illustrated by T5 and T6 in Hachalu variety, respectively. Maketon et al. (2008) have showed that the combined inoculation of *B. subtilis* AP-01 and *T. harzianum* AP-001 resulted in no difference from the fungicides treatment of tobacco leaf spot diseases (chlorothalonil 75%WP and dazomet 5%).

On the other hand, significant difference ($p>0.05$) of shoot and root dry weight was indicated by T4 with 5.07 g and 0.66 g in Ashebeka and 5.76 g and 0.82 g in Hachalu variety, respectively (Table 4.3). The shoot dry weight displayed 56% increase over T1 in Ashebeka variety. In the same pattern, an increase of 61% shoot dry weight over T1 was observed in Hachalu variety. This indicates the influence of chocolate spot on faba bean growth parameters in the absence of bioagents and fungicides. The study indicated the better performance of faba beans in the presence of chocolate spot when treated by T4, followed by T5 and T2 that showed almost similar trend of shoot dry weight increment in both varieties over the respective controls. Muthomi et al. (2007) have found that fungicides (copper oxychloride) application under the presence of biotic stress (fungal) pathogen negatively affected the shoot and root dry weight of common bean. According to Ahemad and Khan (2012), the sole application of fungicide, Tebuconazole, declined the root dry biomass by 34 and 43% above the uninoculated plants at 50 and 80 days after sowing, respectively. Similarly, the dry weight of shoot reduced by 40 and 48%, relative to the control in the same order of days after sowing in green gram (*Vigna radiate* L.).

Table 4.4: The influence of antagonistic isolates (*Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95) applied individually or in a mixture against *Botrytis fabae* infected pot soil and their effects on faba bean (Ashebeka and Hachalu varieties) growth parameters in greenhouse experiment

Treatments	Ashebeka			Hachalu		
	SDW(g)	RDW(g)	R/SR	SDW(g)	RDW(g)	R/SR
T1-Control (B.f only)	3.24^{c}	2.18^{a}	0.46^{a}	3.59^{c}	1.77^{ab}	0.49^{a}
T2-*T. harzianum* AAUT14+B.f	4.52^{ab}	0.64^{b}	0.14^{b}	4.86^{ab}	0.65^{c}	0.13^{b}
T3-*B. subtilis* AAUB95+B.f	4.13^{abc}	1.75^{a}	0.42^{a}	4.45^{ab}	1.85^{ab}	0.42^{a}
T4-*T. harzianum* AAUT14+*B. subtilis* AAUB95+B.f	5.07^{a}	0.66^{b}	0.13^{b}	5.76^{a}	0.82^{bc}	0.14^{b}
T5-MORE 720 WP+B.f	4.15^{abc}	2.13^{a}	0.52^{a}	4.89^{ab}	1.98^{a}	0.40^{a}
T6-ORZEB 80 WP+B.f	4.00^{bc}	2.00^{a}	0.50^{a}	4.68^{ab}	1.87^{ab}	0.40^{a}
CV	0.16	0.44	0.47	0.17	0.44	0.44

B.f-*Botrytis fabae*, SDW-shoot dry weight, RDW-Root dry weight, R/SR-root to shoot ratio, CV-coefficient of variation. Mean values three replication within same columns labeled with same letter (s) of superscript are not different ($p>0.05$) according to Tukey HSD using Post Hoc analysis of Two-Way ANOVA.

The inoculation of *B. subtilis* AAUB95 alone was found to promote growth parameter that was comparable with that of T5. Therefore, this study indicates that as the dual inoculation can provide better growth promotion of faba beans in addition to acting as biological control agents of chocolate spot than single inoculation and fungicide spray. This may be associated with the bioavailability of nutrients sufficiently to the host, and or the commonly shared antagonistic mechanisms in respond to the studied pathogen, reflecting the synergistic effects displayed by the strains (Tang *et al*., 2020).

The root to shoot ratio also varied from 0.13-0.67 in Ashebeka and 0.13-0.49 in Hachalu variety. The lowest root to shoot ratio was showed by T4, whereas the maximum ratio was observed in the treatment that received T1 as a control in both varieties. Bechtaoui et al. (2020) have found a root to shoot ratio of 0.12 in faba beans treated by dual inoculation (PGP30+RhOF57A) of plant growth-promoting rhizobacteria compared to 0.78 of the control. An increase in the root to shoot ratio would indicate that the plant was probably grew under less favorable conditions due to the presence of *B. fabae*. Any factor which improves growing conditions such as favorable weather, fertilization, aeration, or pest control, results in a reduced root-shoot ratio (Harris, 1992).

The nitrogen and phosphorus content analysis showed variability among and between the treatments of this study (Table 4.4). The shoot nitrogen content that varied from 1.42-2.58%, and 0.18-0.34% of phosphorus was recorded by the treatments in Ashebeka variety. In Hachalu variety, 1.43-2.70% and 0.19-0.35% of nitrogen and phosphorus were found, respectively. The highest content of nitrogen and phosphorus was displayed by dual inoculation followed by the single inoculation of bioagents in both varieties. The treatment that received either T2, T3 and or both as dual inoculant showed significant difference ($p<0.05$) compared to the control. This might be due to the ability of the strains in phosphate solubilization as determined in our previous study (under *in vitro* conditions) and supply to the host plant (faba bean). The shoot nitrogen content increased from 40-82% in Ashebeka and 36-88% in Hachalu variety compared to T1.

The phosphorus content increased from 22-88% and 21-84% in Ashebeka and Hachalu variety, respectively, by the treatments. In both cases, the higher percentage of nitrogen and phosphorus was displayed by T4 than the other treatments. Bechtaoui et al. (2020) have also reported as the co-inoculation of plant growth-promoting rhizobacteria retained and improved the phosphorus concentrations from that of 143-230% in faba bean plants compared to the uninoculated and individually inoculated ones. Their finding is very much higher than the present finding, which might be due to the experimental setup utilized by the authors. In their study, the treatments were supplied with either tricalcium phosphate and/or irrigated with potassium phosphate that can be considered as the source of variation from the present study. Elkoca et al. (2007) reported that 7.8% increase of nitrogen content in chickpea treated with co-inoculation of *B. subtilis* and *B. megaterium*. The variation might be associated with the variations of host plants utilized in this

study and/or the studied strains, whereby the *B. subtilis* was combined with *T. harzianum* rather than with beneficial bacterial species of the same genera.

Table 4.5: The effects of single and combined applications of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 to faba bean seeds (Ashebeka and Hachalu varieties) in the presence of *Botrytis fabae* on the nutrient accumulation of shoot using pot experiment

Treatments	Ashebeka		Hachalu	
	N (%)	P (%)	N (%)	P (%)
T1-Control (B.f only)	1.42^{b}	0.18^{b}	1.43^{b}	0.19^{b}
T2-*T. harzianum* AAUT14+B.f	2.17^{ab}	0.22^{ab}	2.00^{ab}	0.25^{ab}
T3-*B. subtilis* AAUB95+B.f	2.37^{ab}	0.24^{ab}	2.33^{ab}	0.26^{ab}
T4-*T. harzianum* AAUT14+*B.subtilis* AAUB95+B.f	2.58^{a}	0.34^{a}	2.70^{a}	0.35^{a}
T5-MORE 720 WP+B.f	1.99^{ab}	0.23^{ab}	1.95^{ab}	0.24^{ab}
T6-ORZEB 80 WP+B.f	2.07^{ab}	0.22^{ab}	2.00^{ab}	0.23^{ab}
CV	0.30	0.42	0.32	0.39

B.f-*Botrytis fabae,* N-nitrogen, P- phosphorus, CV-coefficient of variation. Mean values of three replications in the same column represented by the same letter (s) of superscript indicates no significant difference ($p>0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

4.4 Conclusion and Recommendations

The present study demonstrated that the mixture of antagonistic *T. harzianum* AAUT14 and *B. subtilis* AAUB95 strains as seed coating and foliar spraying reduced the chocolate spot disease incidence and severity of faba beans under greenhouse conditions. Moreover, the co-inoculation resulted in better growth-promoting parameters of faba beans than either *T. harzianum* AAUT14 and or *B. subtilis* AAUB95 application alone. Thus, the treatments need to be further evaluated under field conditions for better confirmation of its potency.

CHAPTER 5

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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Abstract

In Ethiopia, faba bean is mainly grown as food for human consumption. However, foliar fungal disease (chocolate spot) cause appreciable yield loss in the country. The use of fungicides against the disease has offered good results, but the cost rise of fungicides and their negative impact on the ecosystem necessitates the use of biological control agents (BCAs) such as *Trichoderma* and *Bacillus* spp. Thus, this study was conducted to evaluate the performance of single and combined applications of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 as BCAs of chocolate spot on two faba bean varieties (Ashebeka and Hachalu) at Kulumsa Agricultural Research Center. The data were analyzed by SPSS version 24. On Ashebeka variety (Trail 1), a reduction varied from 31-61% for disease incidence and 13-33% for disease severity compared to the control (T1) upon 70 days after sowing (DAS). Upon 90 DAS, the disease incidence and severity reduced to 20-50% and 36-51%, respectively. In co-inoculation, *T. harzianum* AAUT14+*B.subtilis* AAUB95 (T4) reduced the disease incidence and disease severity showing no significance difference ($p>0.05$) with the chemical fungicide, MORE 720 WP (T5) upon 70 and 90 DAS. On Hachalu variety (Trial 2), the microbial inoculants reduced the disease incidence and severity to 28-63% and 17-30% upon 70 DAS, respectively. Likewise, the disease incidence and severity was reduced to 23-51% and 37-54% upon 90 DAS. The AUDPC was ranged from 1586.1-2250.0% in Trial 1 and 1382.0-2454.5% in Trial 2. There was also 62% and 34% increment of hundred seed dry weight and grain yield, respectively over T1 in Trial 1 and 56% and 38%, increase in Trial 2 by T4. *Trichoderma harzianum* AAUT14 (T2) gave 21% and 22% yield increase in Trial 1 and 2, respectively. In

addition, the seed nitrogen content was increased from 33-70% in Trial 1 and 29-62% in Trial 2. Even though the fungicides (T5 and T6) protected the faba bean plants from chocolate spot, there was <10% improvement of seed nitrogen and protein content in both trials. However, seed crude protein enhanced from 33-70% in Trial 1 and 29-66% in Trial 2 by the application of at least one of the bioagents. In conclusion, the mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 or in some cases *T. harzianum* AAUT14 performed best on controlling chocolate spot and increasing yield of faba beans under field conditions.

Keywords: Bioagents; co-inoculation; crude protein, fungicides; harvest index; leaf area; yield

5.1 Introduction

In Ethiopia, faba bean is mainly grown as food for human consumption and it is considered as one of the most important legume crop due to its nutritional value (Samuel Sahile *et al*., 2008). Its seed contains 24-30% of proteins (GRDC, 2017). In addition, faba bean helps to improve soil fertility through biological nitrogen-fixation (BNF). According to Somesagaran and Hoben (1994), it is one of the highest nitrogen fixing crop with an average of 240-325 kg N ha^{-1} yr^{-1}. However, the productivity of faba bean in the country is still below its potential due to biotic and abiotic constraints (Tamene Temesgen *et al.,* 2015). Thus, improving the production of this crop is one of the main objective of the agricultural activity in the country. Fungal pathogens are one of the biotic factors that are responsible for the considerable yield losses of faba bean in many countries (Hebblethwaite, 1983). In Ethiopia, the yield losses of up to 67.5 % was recorded in susceptible faba bean cultivars by chocolate spot disease (Samuel Sahile *et al.*, 2010).

Foliar fungal diseases such as chocolate spot (*Botrytis fabae* and *Botrytis cinerea*), alternaria leaf spot (*Alternaria alternata*), rust (*Uromyces fabae*), and downy mildew (*Peronospora viciae*) are the causative agents of yield losses and its components (Behairy *et al*., 2014). 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 (Hebblethwaite, 1983). The disease is capable of devastating the unprotected faba bean and results in a harmful effect on the plant growth, physiological activities and yield of the crop and sometimes complete crop failures (Saber *et al*., 2009).

The use of fungicides against chocolate spot has offered good results, but the rise in cost 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 (pathogens) of plants and the search for potent microbe is also increasing as potential biological control agents (Deshmukh *et al*., 2010).

Trichoderma and *Bacillus* species are the best microbial members that act as biological control agents of chocolate spot (*B. fabae*) under *in vitro* and *in vivo* conditions (Mater *et al*., 2009). Thus, the application of *Bacillus* and *Trichoderma* species as biological control agents has received much

attention for sustainable agricultural activity in many countries. In addition, these microbes have the ability to produce phytohormones, antifungals, solubilize the insoluble nutrients and provide to the host plants. In faba bean, the mixture of *B. subtilis* and *T. viride* increased the yield by 33% compared to the uninoculated control ones in the presence of faba bean rust infection as biotic stress (Abada *et al*., 2019). Similarly, the application of different *T. harzianum* strains under field conditions resulted in an increase of yield from 8-30% beside acting as the biocontrol agents of chocolate spot (Emeran *et al*., 2006). Coca-Morante and Mamani- Alvarez (2012) have found that seed treatment of the bioagents combined with foliar treatments were more effective for controlling leaf spot of faba bean than foliar spray only.

The combination of *R. leguminosarum* and *T. viride* tag 4 as seed treatment and foliar spray increased the yield by 23% compared to the uninoculated control plants (Saber *et al*., 2009). 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. Although Abd-El-Khair et al. (2018) have showed that field application of *T. harzianum* and *B. subtilis* combined with *Rhizobium* sp. reduced the disease incidence of *F. solani* and increased growth and yield parameters of faba bean, but there is limited information on such treatment on chocolate spot disease with faba bean.

In our previous studies, two strains (*T. harzianum* AAUT14 and *B. subtilis* AAUB95) were found to be effective for their antagonistic property against *B. fabae* (chocolate spot) and plant growth-promoting traits under *in vitro* study (This study Chapters 2 and 3). Moreover, the combination of the strains showed better biocontrol of chocolate spot and improved faba bean growth under *in vivo* (greenhouse) conditions (Chapter 4).

Therefore, the objective of this study was to evaluate the separate and combined inoculation of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 as single and dual inoculation as biocontrol agents of chocolate spot in comparison to fungicides and the growth promoting agents of faba bean under naturally infected field conditions.

5.2 MATERIALS and METHODS

5.2.1 Description of the Study Site

This experiment was conducted at Kulumsa Agricultural Research Center (KARC), at the time of faba bean growing season, June-November, 2018. The center was established in 1966 by government of Ethiopia and the Swedish International Development Agency (SIDA). It is located at 8º2'N and 39º10'E coordinates, having 2200 m above sea level, annual rainfall of 840 mm and covering a total area of 442.7 ha in Tiyo district of Arsi zone at Kulumsa area. Its climatic condition varies from cool highland to semi-arid (agro-ecological zones) and 120-135 days length of different crop growing time. The site is mandated to highland pulse crops mainly faba bean and cereals such as malt barley and wheat research nationally and serves as wheat Center of Excellence for East Africa (Ethiopia, Kenya, Uganda, Tanzania), regionally. This field is naturally infected with *B. fabae*, the causal agent of faba bean chocolate spot.

The dominant soil type of the study area is a vertic Luvisol (IUSS Working Group WRB, 2014) whereas the soil texture is clay loam, and the soil bulk density was 1.25 g cm^3. According to Samuel Lindi et al. (2019) the field capacity and permanent wilting point at the experimental is 33.60% and of 21.8%, respectively, which has the total available water of about 11.80%. The area is characterized by uni-modal rainfall pattern with mean annual rainfall of 809 mm. The mean maximum and minimum air temperature of the study area are 23.08°C and 9.90°C, respectively. The summarized climatic information of Kulumsa area (2015/16-2016/17) is shown below (Table 5.1).

Table 5.1: The Climatic Conditions of Kulumsa area (Samuel Lindi *et al*., 2019)

Year	Months	Rainfall (mm)	Effective RF (mm)	RH (%)	Sun Shine (hrs.)	Tmax (°C)	Tmin (°C)	Wind Speed (m/s)
2015	November	28.40	7.40	58.33	8.04	23.68	12.15	2.20
	December	0.30	-.9.82	62.06	7.56	23.12	11.43	2.16
	January	20.90	2.54	64.58	8.89	24.73	11.95	1.44
2016	February	1.90	-8.86	52.21	7.03	26.47	11.30	2.17
	November	12.20	-2.68	56.65	7.51	23.52	11.20	1.76
	December	0.00	-10.00	55.39	8.96	22.60	10.42	2.35
	January	0.00	-10.00	44.57	8.94	24.08	9.14	2.39
2017	February	29.10	7.46	60.86	6.98	24.67	10.70	1.58

RF-Rainfall, RH-Relative humidity, Tmax-Maximum temperature, Tmin-Minimum temperature and m/s-meter per second.

5.2.2 Sources of Bioagents, Host varieties, and Fungicides

Trichoderma harzianum AAUT14 and *B. subtilis* AAUB95 (antagonistic agents) were obtained from our previous studies. The rhizobial strain, *R. leguminosarum bv*. viciae (FB-1035) was taken from Holleta Agricultural Research Center and the two faba bean (Ashebeka and Hachalu) varieties were obtained from Kulumsa Agricultural Research Center. The two faba bean varieties (Ashebeka and Hachalu) on which the combined application of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 performed best on chocolate spot disease was used for this experiment. Thus, the two faba bean varieties were subjected to field experimental study to further evaluate the single and combined applications of the study strains. The fungicides, viz, MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80 WP (Mancozeb) were bought from local market.

5.2.3 Inocula Preparation

The inoculum of *T. harzianum* AAUT14 was prepared according to Navaneetha et al. (2015). A 5 mm mycelial disc was inoculated on fresh potato dextrose agar (PDA) and incubated at 25±2°C for five days. After incubation, 10 mL sterile water was added to the plates, scrapped slightly using the suspension and filtered through two layers sterilized gauze. the spore suspension was collected into flasks and adjusted to 2.5×10^5 spore mL^{-1} using haemocytometer (Mohammed *et al.*, 1994). *Bacillus subtilis* AAUB95 was cultured in 100 mL flasks containing 40 mL nutrient broth. The flasks were incubated at room temperature on shaker at 130 rpm for 48 hrs. and $1x10^9$CFU mL^{-1} of cells were utilized for the study (Sharga, 1997).

5.2.4 Seed Coating, Foliar spray of the Bioagents and Fungicides

Faba bean seeds, were washed with tap water, surface sterilized by 3% sodium hypochlorite for 1 min and rinsed in distilled-sterilized water thoroughly. Seeds were air dried over the sterilized filter paper under fume hood and checked for germination on Petri plate. Seeds were treated with *T. harzianum* AAUT14 having 2.5×10^5 spore mL^{-1} (Mohammed *et al.*, 1994), *B. subtilis* AAUB95 with $1x10^9$CFU mL^{-1} (Sharga, 1997) and *R. leguminosarum bv, viciae* (FB-1035) containing $1x10^9$ CFU mL^{-1} (Benidire *et al*., 2017) as seed coating using 10% carboxyl methyl cellulose (CMC) each at a rate of 10 mL/kg of seed, air dried and sown directly (Zaim *et al*., 2018). In addition to seed coating prior to sowing, foliar spraying of the developed plants with the same bioagents (*T. harzianum* AAUT14 and *B. subtilis* AAUB95) was done two times on the 35^{th} and 55^{th} days after sowing with 3 mL/plant (Saber *et al*., 2009) and the fungicides, MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80 WP (Mancozeb) were applied to faba beans according to the manufacture's instruction given on the packs.

5.2.5 Experimental Layout and Treatments

The plots were prepared with 3.2 m^2 (4 x 0.8 m) area, 60 cm distance between plots, 40 cm distance between rows, 4 m length and 1.5 m 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 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 EIAR (2018). All the agronomic practices were done manually with the involvement of human power. Accordingly, the following treatments were allocated in the study: -

T1-Control (*B. fabae* only)
T2-*T. harzianum* AAUT14+*B. fabae*
T3- *B. subtilis* AAUB95+*B. fabae*
T4- *T. harzianum* AAUT14+*B. subtilis* AAUB95+*B. fabae*
T5- MORE 720 WP+*B. fabae*
T6- ORZEB 80 WP+*B. fabae*

5.2.6 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 stages of symptoms development. Disease severity was expressed as percent of affected leaf based on symptoms appeared according to Gullino et al. (2017) 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 the infected leaves on the basis affected areas` disease strength through visual observations. An area under disease progress curve (AUDPC) in percentage of development stage unit (%DSU) was calculated according to Hanounik and Hasanain (1986).

%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 (%DSU).

Disease reduction percentage (%R) per treatment was calculated according to Zaim et al. (2018) 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.

5.2.7 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 also calculated by considering diseased pods (showing symptom of chocolate spot). The succeeding model proposed by Peksen (2007) was used to calculate an area of leaf using randomly taken leaf samples from each treatments.

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

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

On harvesting, 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 El-Naim et al. (2010).

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

5.2.8 Seed Nitrogen Content analysis and Crude Protein Estimation

Seed nitrogen content was analyzed according to Kjeldahl method. Two hundred milligram (200 mg) of dried seed sample was taken in a 100 mL Kjeldahl flask, 5 mg of salt mixture (potassium sulphate, cupric sulphate and selenium powder mixed in the ratio of 50:10:1) was added with 3 mL of concentrated sulphuric acid, followed by digestion. After digestion, 10 mL of distilled water was added. The distillate was collected in a conical flask having 10 mL of 4% boric acid and 3 drops of mixed indicator (0.3 g bromocresol green and 0.2 g 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 (Jones,1941).

$$\text{Nitrogen (\%)} = \frac{\text{Sample titre - blank titre} \times \text{Hot HCl} \times 14 \times 100}{\text{Sample weight} \times 1000}$$

5.2.9 Statistical Analysis

The data were analyzed by General Linear Model (GLM) univariate analysis of variance of Two Way-ANOVA using randomized completely block design (RCBD). The interaction among disease, hosts and days of disease assessment was conducted by Three-Way ANOVA. Mean values were separated by Tukey HSD analysis at $\alpha = 0.05$ of SPSS version 24 and all the values were considered significant at $p<0.05$.

5.3 Results and Discussion

The treatment exhibited different levels of chocolate spot disease incidence and severity along with an area under disease progress curve as a function of days after sowing in Trial 1 (Ashebeka) variety (Table 5.2). In a reduction that varied from 31-61% and 13-33%, was recorded for disease incidence and disease 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.

This shows the existence of variation among and between the treatment applied to each experimental unit in controlling the pathogen and its respective disease (chocolate spot). The dual application of bioagents, *T. harzianum* AAUT14+*B.subtilis* AAUB95 (T4) showed reduction of disease incidence and disease severity showing no significance difference ($p>0.05$) with the chemical fungicides, MORE 720 WP(T5) up on 70 and 90 days of sowing. This may indicate the efficiency of the strains to be used as the effective of fungicide (T5) in biological control of chocolate spot disease depending on their antagonistic property. On the other hand, *T. harzianum* AAUT14 (T2) performed better than *B. subtilis* AAUB95 (T3) that showed less efficacy in this experiment. Ramadan and El-Kholy (2013) also reported that the effectiveness of *Trichoderma* spp. compared to *Bacillus megaterium* in controlling chocolate spot under field study. Abeysinghe (2007) have also reported the better efficacy of *T. harzianum* than *B. subtilis* in controlling faba bean fungal pathogen, *F. solani* in common beans and chickpea (Zaim *et al*., 2018). This could be attributed to the mechanisms through which *T. harzianum* AAUT14 or *B. subtilis* AAUB95 antagonized *B. fabae*. The same trend of performance was also displayed by the strain under the greenhouse conditions. 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 that is comparable with the chemical fungicides (T5 and T6). The combination of *T. harzianum* T5- and *B. subtilis* Bs1 strains, provided the best reduction *Fusarium* infection of disease severity and incidence in comparison to the individual treatments in chick pea (Zaim *et al*., 2018). In this regard, Pierson and Weller (1994) demonstrated that the efficiency of biological control agents in mixtures was related to complementary modes of actions by the combined microorganisms.

Table 5.2: The effect of single or dual applications of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on faba bean (Ashebeka) chocolate spot (*Botrytis 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.f only)	23.80^{a}	-	40.00^{a}	-	58.00^{a}	-	75.00^{a}	-	2250.0^{a}
T2-*T. harzianum* AAUT14+B.f	14.00 ^{bc}	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^{d}	55	28.00^{d}	30	29.26^{e}	44	38.00^{e}	47	1660.1^{d}
T5-MORE 720 WP+B.f	9.24^{d}	61	27.00^{e}	33	28.89^{e}	50	37.11^{e}	51	1586.1^{e}
T6-ORZEB 80 WP+B.f	11.59^{cd}	51	28.31^{d}	29	32.26^{d}	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 ($p>0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

The bioagents and chemical treatments reduced the disease incidence and severity on the Hachalu variety in trial 2 (Table 5.3). The bioagent reduced disease incidence and severity in the range of 28-55%% and 17-37% upon 70 days of sowing. Likewise, the disease incidence and severity was reduced to 23-46% and 37-48% in 90 days of sowing by one of the bioagents applied, respectively. This shows as the application of bioagents reduced the disease incidence and severity compared to the control (T1) in both trials. Similarly, Ramadan and El-Kholy (2013) reported the disease severity reduction of chocolate spot in faba beans treated by *Trichoderma* and *Bacillus* spp. compared with the untreated faba bean plants. The T4 inoculated plants showed the highest reduction in disease incidence especially at the latter days (90 days) of treatment (44%) compared to T5(50%) and T6(44%) treated plants. This indicates as the performance of T4 was in between the chemical fungicides T5 and T6 used in the study, in which the same trend of performance was observed in trial 1.

In addition, AUDPC that ranged from 1586.1-2250.0% and 1382.0-2454.5% was shown by the different treatments in trial 1 (Table 5.2) and trial 2 (Table 5.3), respectively. The maximum AUDPC observed was 2250.0% by T1 in trial 1 and 2454.5% in T1 of trial 2. Dagne Kora et al. (2017) have reported that fungicides unsprayed faba bean, with 1817%,1476%, 1467% and 1716% of AUDPC on Sinana local, Shallo, Mosissa and Walki varieties of faba bean, respectively. Their finding is different from this study which might be caused by the difference in the varieties used in the study and or due to the inoculum size of the pathogenic fungi (*B. fabae*) found in the study fields. Nevertheless, in this study T4 demonstrated 1660.1% and 1478.5 % of AUDPC in trial 1 and 2, respectively. Thus, this indicates as the chocolate spot symptom development was significantly influenced by the dual treatment compared to the single treatments.

Table 5.3: The effect of single or dual applications of *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on faba bean (Hachalu variety) chocolate spot (*Botrytis 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.f only)	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^{bc}	34	39.25^{bc}	40	1787.67^{c}
T3-*B. subtilis* AAUB95+B.f	16.00^{ab}	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^{cd}	55	25.30^{d}	37	30.17^{cd}	46	34.00^{cd}	48	1478.5^{de}
T5-MORE 720 WP+B.f	8.15^{d}	63	24.00^{e}	40	27.20^{d}	51	30.20^{d}	54	1382.0^{e}
T6- ORZEB 80 WP+B.f	11.00^{c}	50	25.40^{d}	37	33.16^{cd}	40	36.50^{bc}	44	1508.0^{d}
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 ($p>0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

In addition, the interaction analysis among the chocolate spot disease level (disease severity and disease incidence), hosts and days of disease assessment was significantly different ($p<0.05$) for both faba bean varieties (Table 5.4).

Table 5.4. An interaction analysis among the chocolate spot disease level (disease severity and disease incidence), hosts and days of disease assessment by Three- Way ANOVA

Tests of Between-Subjects Effects

Dependent Variable: chocolate spot disease

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	11246.100[a]	7	1606.586	27.481	.000	.586
Intercept	146421.660	1	146421.660	2504.536	.000	.948
days	8176.530	1	8176.530	139.859	.000	.507
disease	2147.164	1	2147.164	36.727	.000	.213
hosts	290.788	1	290.788	4.974	.027	.035
days * disease	340.003	1	340.003	5.816	.017	.041
days * hosts	33.476	1	33.476	.573	.451	.004
disease * hosts	12.930	1	12.930	.221	.639	.002
days * disease * hosts	245.210	1	245.210	4.194	.042	.030
Error	7950.912	136	58.463			
Total	165618.672	144				
Corrected Total	19197.012	143				

a. R Squared = .586 (Adjusted R Squared = .565)

Application of both antagonists individually or in combination not only controlled chocolate spot, but also promoted plant growth. In this aspect, the treatments indicated variability in leaf area that derived from the respective leaf length and width of faba beans (Table 5.5). All the treatments showed significant difference ($p<0.05$) with the untreated control in both trials, in which the antagonist inoculated faba bean plants (either separately and or together) showed the largest leaf area than the fungicides (T5 and T6) treated once. An area of 68.95 cm^2 was displayed by T4 and followed by T2 that showed 54.32 cm^2 in trial 1. Correspondingly, in trail 2, 54.14 cm^2 and 50.02 cm^2 of leaf area was shown by T4 and T2, respectively. This may reveal the extra role of bioagents 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 the best growth and productivity in crops (Peksen, 2007).

Table 5.5: The effect of single and dual application of antagonistic *Trichoderma harzianum* AAUT14 and *Bacillus 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.f only)	8.30^{d}	3.37^{c}	20.00^{e}	8.71^{c}	3.23^{c}	20.11^{c}
T2-*T. harzianum* AAUT14+B.f	13.50^{a}	5.80^{ab}	54.32^{ab}	13.50^{a}	5.55^{a}	50.02^{a}
T3-*B. subtilis* AAUB95+B.f	13.00^{ab}	5.77^{ab}	49.05^{bc}	13.00^{a}	5.46^{a}	48.33^{a}
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^{de}	12.81^{ab}	4.72^{ab}	33.30^{b}
T6- ORZEB 80 WP+B.f	11.66^{bc}	4.60^{bc}	37.23^{cd}	11.00^{b}	4.33^{b}	36.45^{ab}
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 ($p<0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

Moreover, 120 days after sowing, all the treatments showed significantly different ($p<0.05$) number of pods and chocolate spot uninfected pods (healthy pods) compared to T1 in both trials as shown below (Table 5.6). This may indicate the potential of the treatments to control chocolate 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 followed by T2 showed 70.40 and 78.86% in trial 1 and 2, respectively. *Trichoderma harzianum* AAUT14 (T2) showed the same trend of protecting faba bean plants from chocolate showing no significant variation ($p>0.05$) with T6 in both varieties (trial 1 and 2). Additionally, the combination of T2 with T3, T4 resulted in a good performance of protecting faba bean pods from chocolate spot following T5 (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 biofungicides in addition to growth-promoting agents of faba bean.

On the other hand, pod number and seeds per pod was 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 followed by T2. Furthermore, T2 showed a comparable result with T5. However, T3 indicated less performance in both trial of this study which might be dealt with potency of the strain when inoculated lonely. Abd-El-Khair et al. (2018) and Shaban and El-Bramawy (2011) have reported that 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.

Table 5.6: The effect of single and dual applications of antagonistic *Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95 on faba bean pod number and symptoms of chocolate spot (*Botrytis 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^{f}	10.07^{d}	1.83^{c}	40.07^{e}
T2-*T. harzianum* AAUT14+B.f	13.40^{b}	2.57^{a}	70.40^{d}	14.17^{b}	3.10^{a}	78.86^{c}
T3-*B. subtilis* AAUB95+B.f	12.00^{c}	2.27^{b}	60.52^{e}	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 80 WP +B.f	11.00^{cd}	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 replications within same columns labeled with same letter (s) of superscript are not significantly different ($p>0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

Following harvest, both the fungicides and bioagents' treatment showed significant difference ($p<0.05$) with respect to yield and yield related parameters from that of control (T1) in both trials (Table 5.5). In the same way, El-Banoby et al. (2013) reported that faba bean plants treated with the fungicide, Diathane M45 (Mancozeb) and biocides, *B. subtilis* and *T. harzianum* gave the highest seed yield/plot, hundred seed weight and the grain yield as compared with the uninoculated control plants. In the present study, the treatment, T4 resulted significant variation ($p<0.05$) to all the treatments with regard to the considered parameters.

In T4 received experimental unit, there was 62 and 34% increment of hundred seed dry weight and grain yield estimate in hectare over T1, respectively in trial 1. The same treatment showed 56 and 38%, increase of hundred seed dry weight and grain yield estimate, respectively in trial 2 (Table 5.7). However, in both cases, the fungicides (T5 and T6) treated plants showed a comparable result to that of T2 in increasing the seed dry weight and yield in the trials. Faba bean plants sprayed with the mixture of *B. subtilis* and *T. viride* increased the yield by 33% compared to the unsprayed ones in the presence of faba bean rust infection as biotic stress (Abada *et al*., 2019). *Trichoderma harzianum* AAUT14 (T2) showed similar trend of better faba bean yield increment following the combined inoculation (T4). The same treatment illustrated 21% and 22% yield increase in trial 1 and 2, respectively. The strains of different *T. harzianum* strains under field conditions resulted an increase of faba bean yield from 8-30% beside acting as the biocontrol agents of chocolate spot (Emeran *et al*., 2006). Likewise, *T. viride* tag4 mixed with *R. leguminosarum* increased the yield of faba beans by 23% compared to the control plants (Saber *et al*., 2009).

In this study, T4 showed no significance difference ($p>0.05$) 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 acting as biofertilizers through overcoming the influence chocolate spot induced by *B. fabae*. Abd-El-Khair et al. (2018) reported that the

combined treatments of *T. harzianum* with *R. leguminosarum* increased the plant growth parameters viz, mean seed dry weight and grain (seed) yield of faba bean in the presence of biotic stress induced by *Fusarium* sp. in field study.

Table 5.7: The effect of single and dual applications of antagonistic (*Trichoderma harzianum* AAUT14 and *Bacillus subtilis* AAUB95) on yield parameters, seed nitrogen and crude protein contents of faba bean (Trial 1-Ashebeka and Trial 2-Hachalu variety) in field experiments

Treatments (Trial-1)	Parameters					
	HSDW (g)	Biomass (kg/ha)	GYE (kg /ha)	HI (%)	SNC (%)	SCPC (%)
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 80 WP +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 80 WP +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 ($p < 0.05$) according to Tukey HSD analysis of Two-Way ANOVA.

Furthermore, harvest index was higher than T1 in both trials that received one of the treatments, which may show the effect of either single and or dual application of the antagonistic strains on faba bean yield improvement. A 14-56% and 23-57% of harvest index increase was exhibited in trial 1 and trial 2, respectively by at least one of the employed treatments (Table 5.5). *Trichoderma harzianum* AAUT14+*B. subtilis* AAUB95 (T4) gave 4391.45 kg/ha and 4378.12 kg/ha followed by T2 with 3764.58 kg/ha and 3654.17 kg/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 found by the treatment that showed the maximum yield estimates. Ashenafi Mitiku and Mekuria Wolde (2015) have also reported a higher grain yield, 4886.8 kg/ha in Shallo and 4362.2 kg/ha in Mosissa variety of faba beans with the highest value of harvest index, 45 and 43%, respectively.

Post-harvest, the seed nitrogen and crude protein content indicated variation from the uninoculated faba bean plants (Table 5.7). 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 negligible improvement of seed nitrogen content in both trials over T1 (<10%). Similarly, the crude protein of seed increased from 36-70% in trial 1 and 31-66% in trial 2 by the application at least one of the bioagents. Similar to the seed nitrogen content, the fungicides showed insignificant ($p>0.05$) improvement of seed crude protein content 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 of plant beneficial microbes (bacterial and fungal) to overcome the stress and their effect on the nutritional status of faba bean seed. The improvement of seed nitrogen content and crude protein can be attributed to nitrogen-fixation, solubilization of phosphorus and production of phytohormones (Rokhzadi *et al*., 2008). 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 better accumulation of proteins and carbohydrates (Mahmoud *et al*., 2004). Even though the faba beans seed protein was reported to be 24-30%, the protein content of less than 24% was detected in this study. This might be associated with the effects of the bioagents and

or the variation in the genotypes of the faba bean varieties used in the study. On the other side, the minimum seed protein content was seen in T1 of both varieties (trials) as compared to the other treatments, that might be correlated to the negative impact of chocolate spot on the nutrient accumulation of faba bean seeds even postharvest.

5.4 Conclusion and Recommendations

In the present study, the mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 or in some cases *T. harzianum* AAUT14 alone performed best on faba bean growth and yield parameters. The study, demonstrated also the future use of these antagonistic microorganisms for controlling chocolate spot of faba bean. Therefore, based on this finding it could be recommended that these isolates should be studied with the combination of chemical fungicides for their compatibility and so as to utilize as an integrated disease management system. Thus, the combination can be an input for faba bean production along with MORE 720 WP (Mancozeb+Cymoxanil) and ORZEB 80 WP (Mancozeb).

CHAPTER 6: General Conclusion and Recommendations

6.1 Conclusion

This study indicated the potential of rhizosphere bioagents (*Trichoderma* and Rhizobacterial isolates) to control *Botrytis fabae*, using dual culture, culture filtrates of different concentrations, detached leaf assay and sealed plate method under *in vitro* conditions. In addition, the isolates were found to exhibit different plant growth promoting and antagonistic properties. From the rhizobacterial species *Bacillus* and *Serratia* species were the most influential group against *B. fabae* with an extra potency of plant growth-promoting traits (phosphate solubilization, indole-3-acetic acid, ammonia and antagonistic features (hydrogen cyanide and lytic enzyme production).

Bacillus subtilis AAUB95 was the most potent isolate that showed the best efficiency to control *B. fabae* in both the dual and culture filterate assay evaluated in different days of incubation. The strain also showed almost all the antagonistic properties checked in this study together with growth promoting features. Following *B. subtilis* AAUB95, *S. nematodiphila* showed good antagonistic ability against the *B. fabae* together with the antagonistic and growth-promoting properties. On the other hand, this study revealed uncommon *Trichoderma* species, *T. afroharzianum*, *T. tomentosum*, and *T. orientale* that associate with faba beans. These isolates were also rarely reported as the biological control agents of *B. fabae*. However, of all the identified *Trichoderma* species, the frequently reported biological control agent, *T. harzianum* (*T. harzianum* AAUT14) was the most effective strain against *B. fabae* under *in vitro* conditions using dual culture assay. The strain also showed consistency in the inhibition of *B. fabae* as a function of incubation times, production of ammonia and displayed the well-known antagonistic properties (mycoparasitism/hyphal coiling.

Under the greenhouse condition, the co-inoculation of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 showed better control of *B. fabae* following fungicides, MORE 720 WP with equivalent or comparable efficiency to fungicides, ORZEB 80W. However, the separate application of *T. harzianum* AAUT14 performed better than *B. subtilis* AAUB95. Furthermore, the combined inoculation boosted the shoot dry weight and the nutrient (nitrogen and phosphorus) accumulation

in the dry faba bean shoots. Similarly, under field conditions, the co-inoculation resulted better growth and yield parameters in faba beans in addition to acting as biological control agents of chocolate spot under naturally infected conditions. A significant reduction ($p<0.05$) of disease severity, incidence and area under disease progress curve and yield improvement was noted as compared to the uninoculated control plants. The single and dual applications of the strains gave better yield and yield related parameters than the fungicides. Thus, it can be concluded that the mixture of *T. harzianum* AAUT14 and *B. subtilis* AAUB95 are useful to protect faba bean from chocolate spot grown in naturally infected field conditions when combined with *Rhizobium* species than fungicides spray only.

6.2 Recommendations

Based on the present study, we would like to recommend the followings: -

- ✓ The mechanisms of interaction that led to better performance when inoculated together should be studied.
- ✓ The compatibility of the two strains have to be studied with fungicides of different concentration so that they can be utilized in integrated disease management (IDM).
- ✓ The strains should be further validated for their potential to be used with different varieties under different field conditions infected with chocolate spot and other diseases such as faba bean rust.

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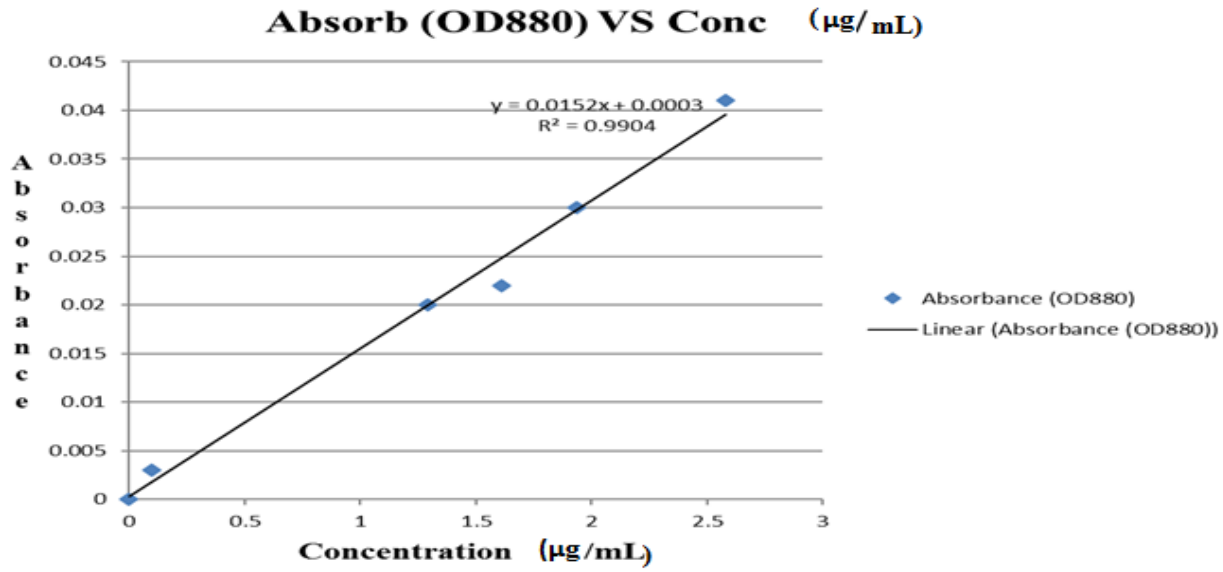
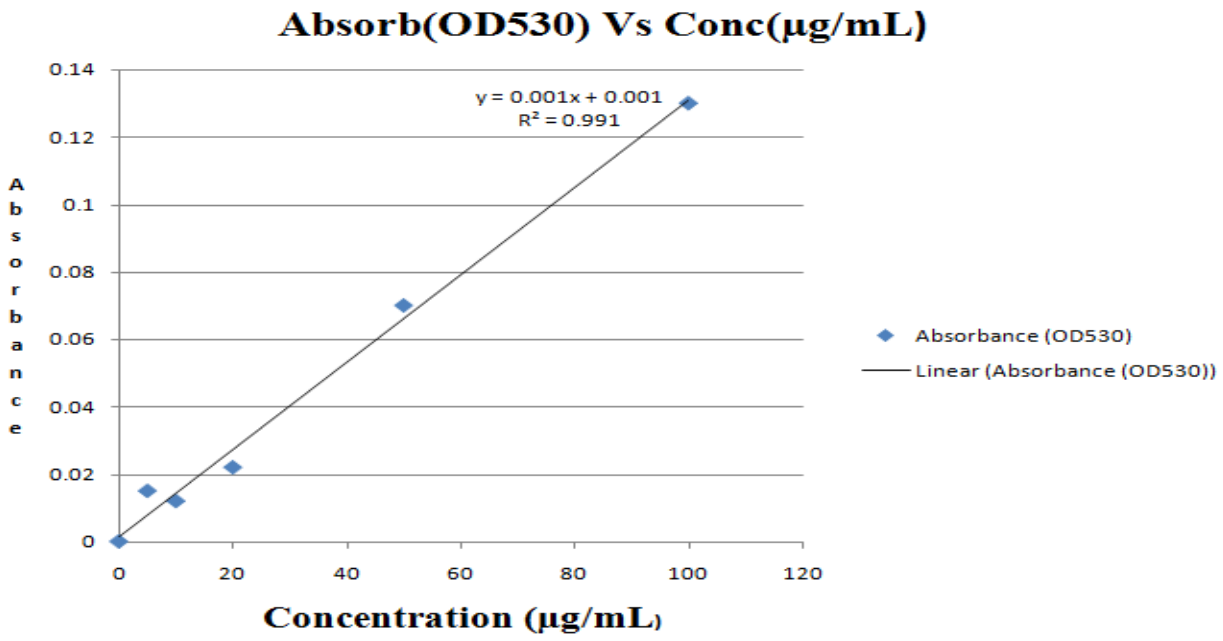
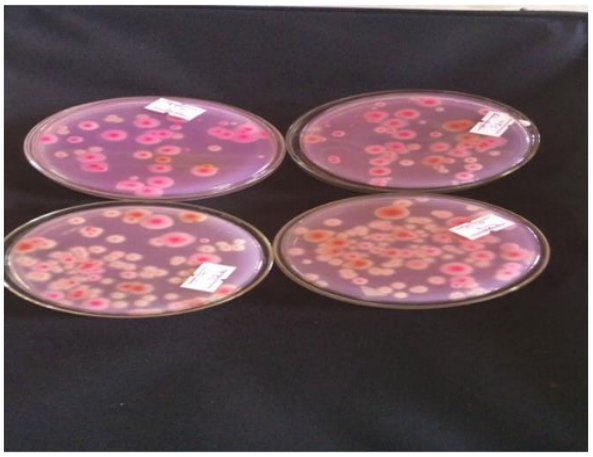
Appendix 1: Standard curve used for the quantification of soil samples phosphorus (PPM), plant materials (%) at 880nm and phosphate solubilizing potentials of bacterial and *Trichoderma* isolates (µg/mL)



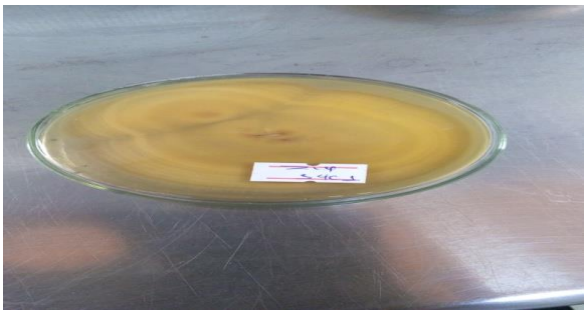
Appendix 2: Standard curve used for the quantification of Indole-3-acetic acid (µg/mL) at 530nm for both the bacterial and *Trichoderma* isolates

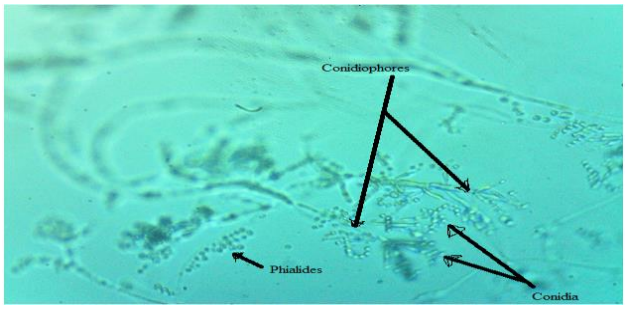


Appendix 3: Representative colonies of *Trichoderma* on rose Bengal agar (RBA) plates on 5-7 days of incubation

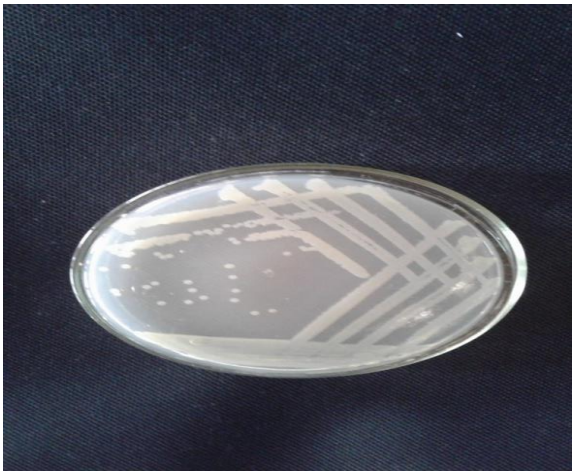


Appendix 4: Morphological and microscopical observation of *Trichoderma* isolates

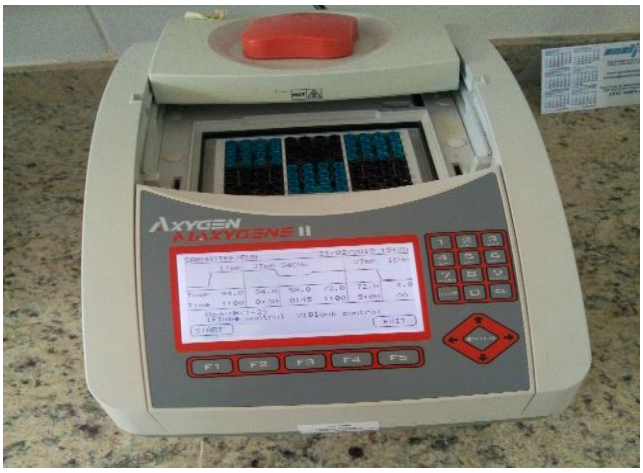




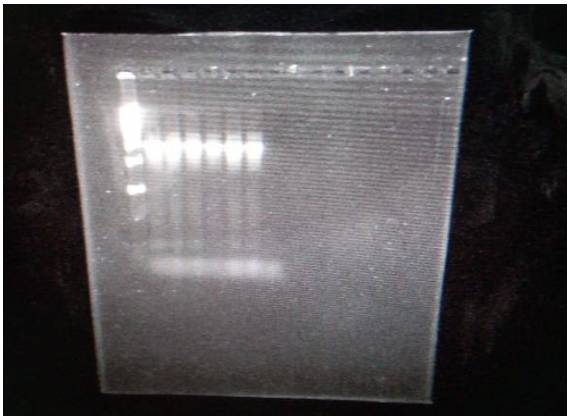
Appendix 5: Bacterial colony on Petri plate (a) , PCR , agarose gel electrophoresis of extracted DNA (c) and the PCR products



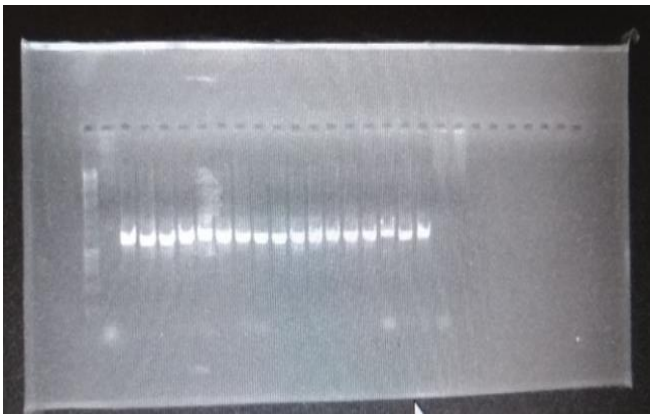
a



b



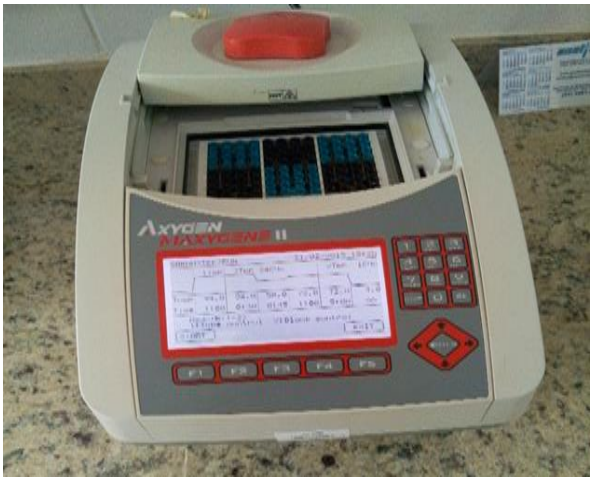
c



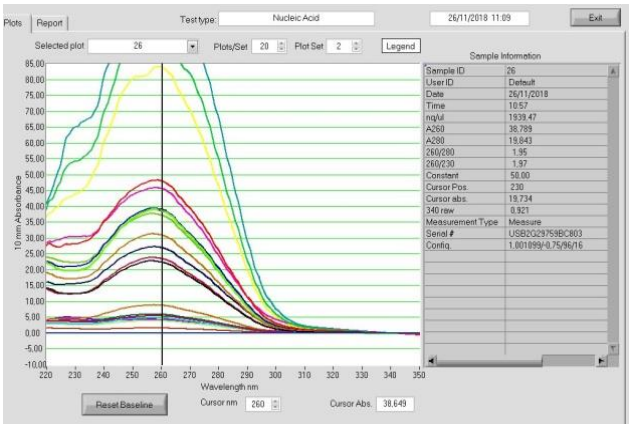
d

Appendix 6: *Trichoderma* spp grown in nutrient broth (a) , PCR , quantification of the extracted DNA througn Nano drop (c) and the PCR products

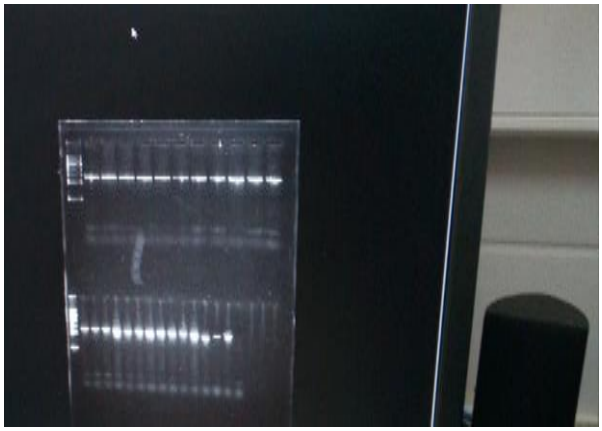
a



b

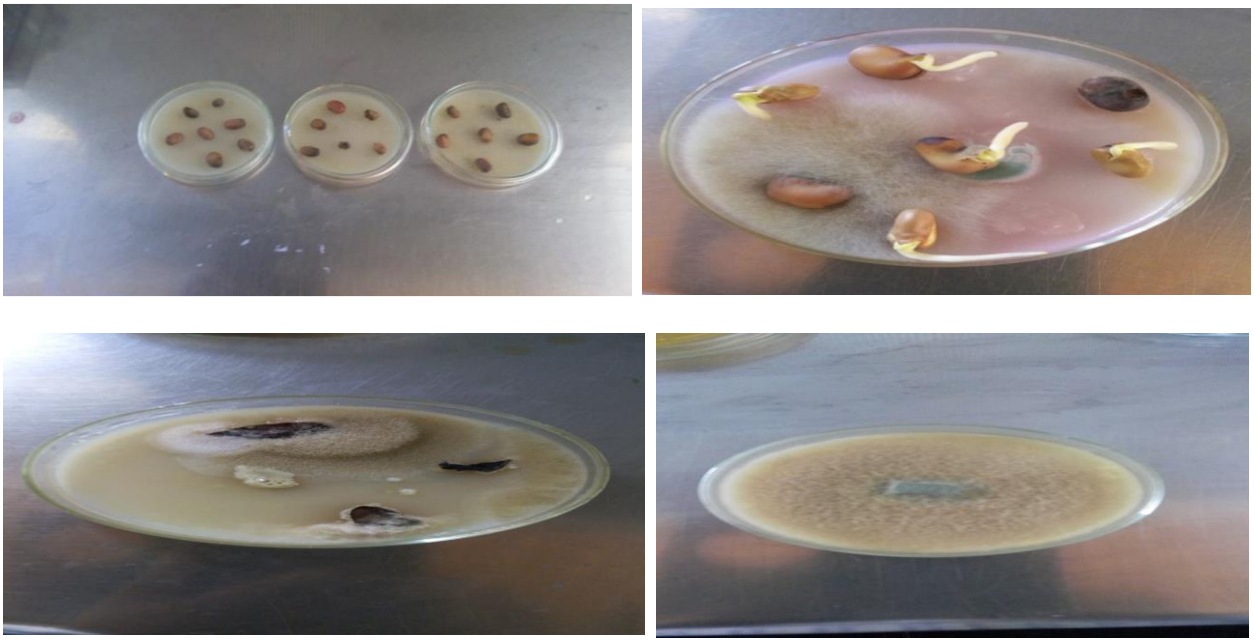


c

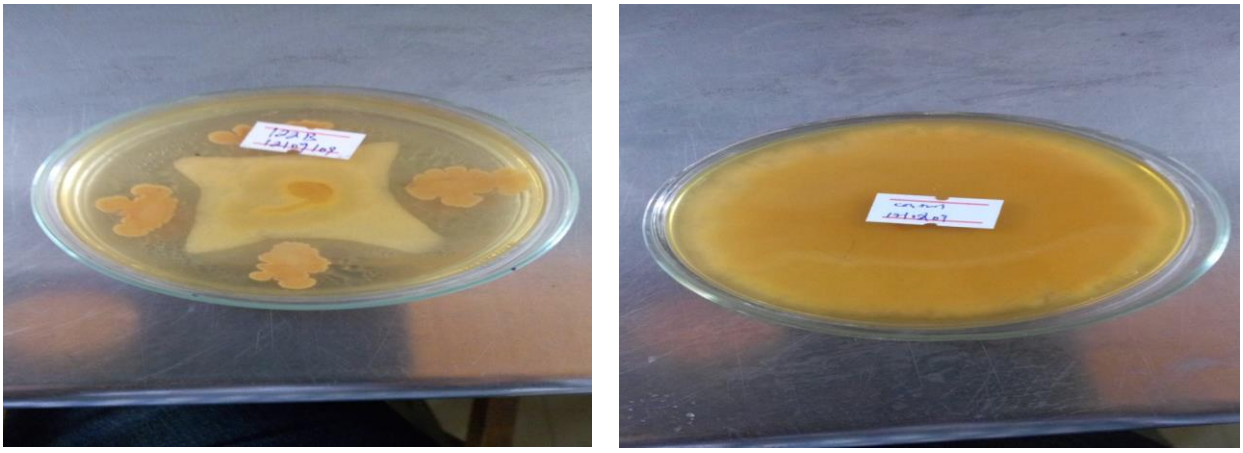


d

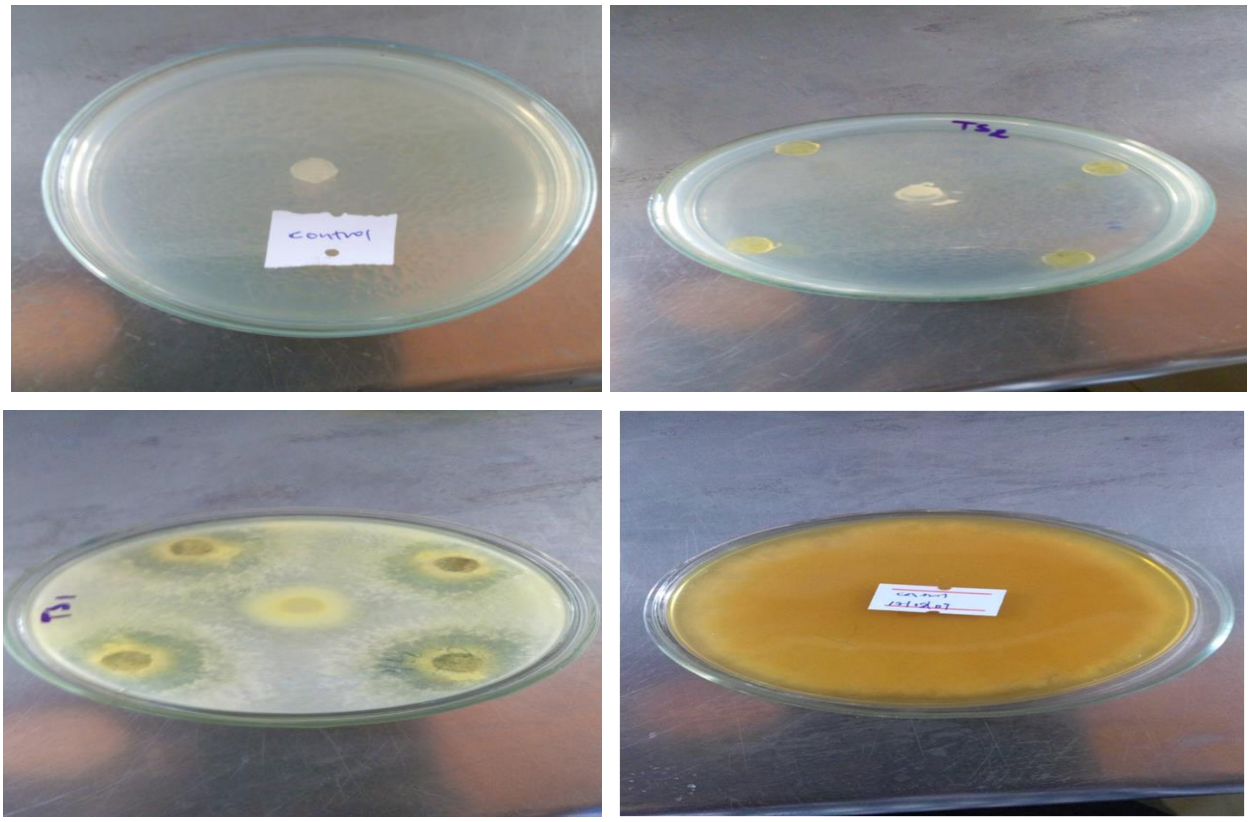
Appendix 7: Isolation of *Botrytis fabae* (test pathogen) from faba bean seeds and leaves samples using faba bean dextrose agar (FDA) Medium



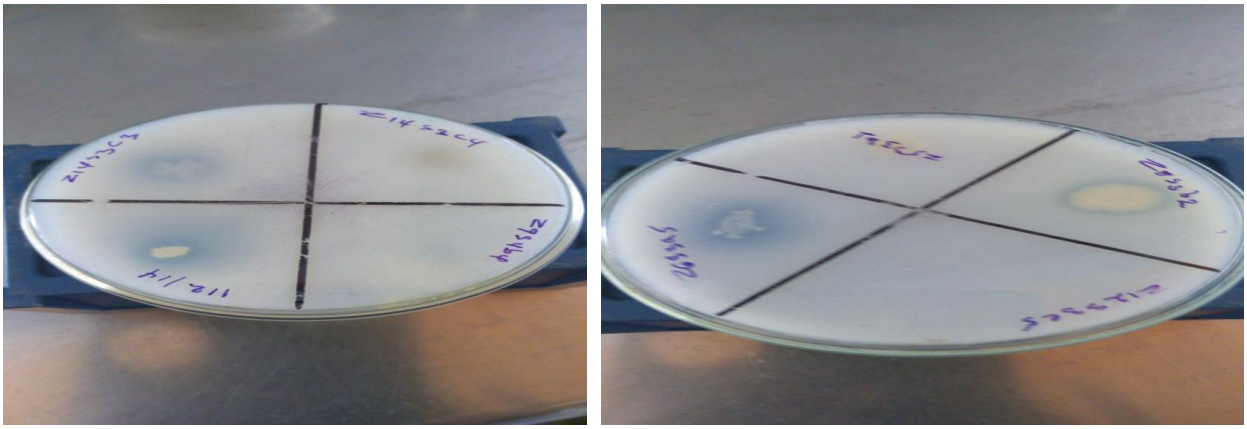
Appendix 8: Screening of bacterial isolates using 10% sucrose amended potato dextrose agar through dual culture assay method

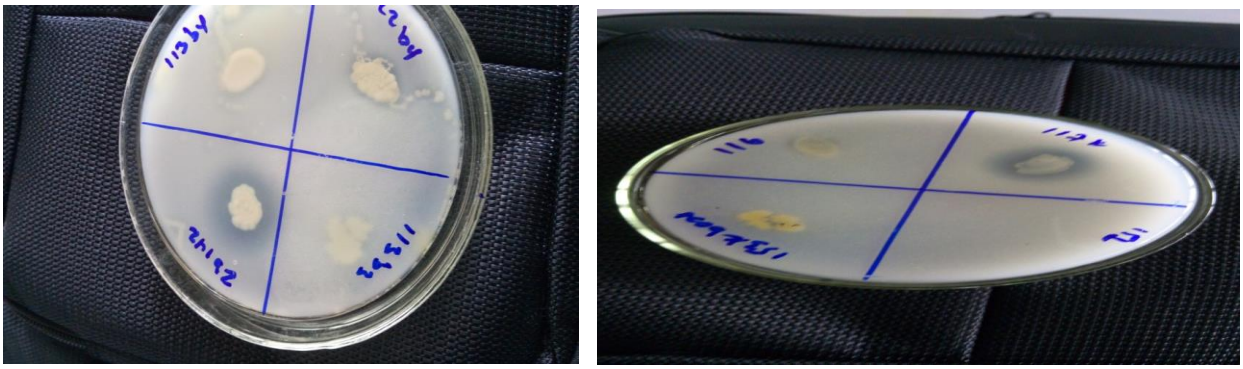


Appendix 9: Screening of *Trichoderma* isolates against *Botrytis fabae* Sard using potato dextrose agar through dual culture assay method

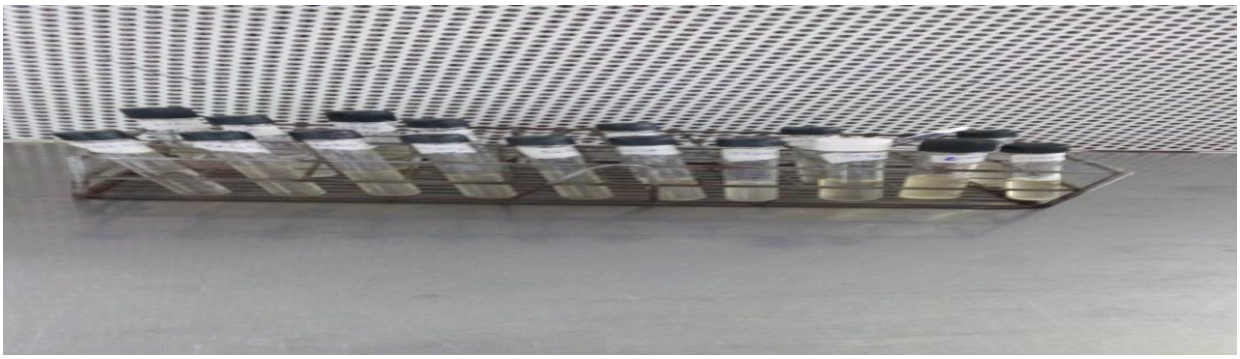


Appendix 10: Tricalcium Phosphate (TCP) solubilizing potential of the different bacterial isolates using Pikovskaya's agar plate





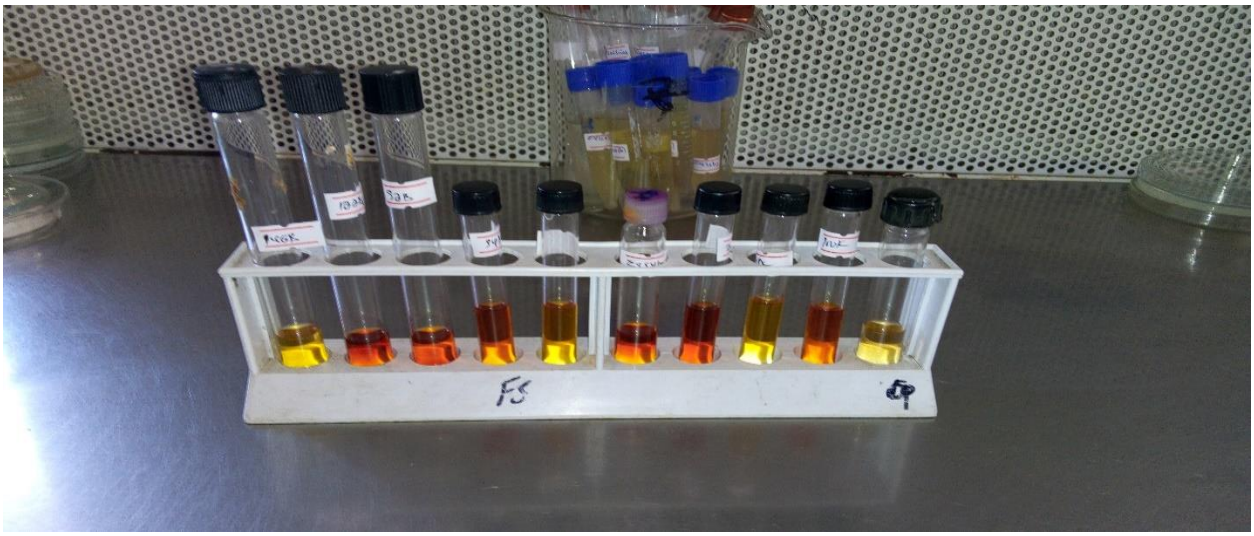
Appendix 11: Test for ammonia production ability by the different bacterial isolates, before the addition of Nessler's reagent to the culture (a) and after the addition of Nessler's reagent to the culture (b)



(a)

(b)

Appendix 12: Test for indole-3-acetic acid production ability by the different bacterial isolates



Appendix 13: Field study experimental layout