ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Amin Mohammed YONES

IDENTIFICATION, CHARACTERIZATION AND BIOCONTROL OF *Botrytis* spp. CAUSING FABA BEAN CHOCOLATE SPOT USING *Trichoderma harzianum*

DEPARTMENT OF PLANT PROTECTION

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ABSTRACT

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Eighteen isolates of *Botrytis* spp., as well as 20 isolates of *Trichoderma* spp., were identified from faba bean and field pea fields in Adana and Mersin provinces. In addition, eight isolates of *Botrytis* strain, collected from faba bean leaves in Ethiopia, were included for molecular study. All fungal isolates were purified using a single spore method. *Botrytis* spp. collected in Adana was identified as *B. cinerea* while all isolates of *Botrytis* collected from Ethiopia were identified as *B. fabae* based on morphological features as well as phylogenetic analysis using two primers (ITS5/ITS4 and Bt2a/Bt2b). All fungal bioagents were identified as *T. harzianum* based on the internal transcribed spacer (ITS5/ITS4) and translation elongation factor (tef71/tef997) primers. The optimum temperature growth ranges for *B. cinerea* and *T. harzianum* isolates were 15-20 °C and 25-30 °C, respectively. From the pathogenicity test experiment, Bc7 and Bc8 were found to be the most virulent among screened *B. cinerea* isolates. All isolates of *T. harzianum* showed promising results in reducing faba bean chocolate spot epidemics both under *in vitro* and *in vivo* conditions. However, the efficacy was variable among the isolates tested.

Keywords: Antagonistic assessment, β-tubulin, EF1-α, ITS

ÖZ

DOKTORA TEZİ

BAKLA ÇİKOLATA LEKE HASTALIĞI ETMENİ *Botrytis* spp.'NIN TANIMLANMASI, KARAKTERİZASYONU VE *Trichoderma harzianum* ILE BIYOLOJIK MÜCADELEDE

Amin Mohammed YONES

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Adana ve Mersin illerinde baklada Çikolata Leke Hastalığı etmeni olarak 18 *Botrytis* spp. izolatı, biyolojik ajan olarak 20 *Trichoderma* spp. izolatı hem bakla hem de bezelye tarlalarından izole edilmiştir. Ayrıca Etiyopya'daki bakla yapraklarından saflaştırılan *Botrytis* spp.'ye ait sekiz izolat moleküler çalışmaya dahil edilmiştir. Tüm fungus izolatları tek spor yöntemi kullanılarak saflaştırılmıştır. İki farklı primer seti (ITS5/ITS4 ve Bt2a/Bt2b) kullanılarak yapılan filogenetik analizler ve morfolojik özelliklere göre Adana'da izole edilen *Botrytis* spp, *B. cinerea* olarak tanımlanırken, Etiyopya'dan gelen *Botrytis* izolatları, *B. fabae* olarak tanımlanmıştır. Fungal biyo-ajan olarak kullanılan *Trichoderma* spp. izolatlarının tümü ITS5/ITS4 EF1-α (tef71/tef997) primer ile elde genlerin filogenetik analizlerine göre *T. harzianum* olarak tanımlanmıştır. *B. cinerea* ve *T. harzianum* izolatlarının büyümesi için optimum sıcaklık aralıkları sırasıyla 15-20 ° C ve 25-30 ° C olarak saptanmıştır. Patojenite testi deneyinde, Bc7 ve Bc8 *B. cinerea* izolatları en virülent olarak saptanmıştır. baklada **ç**ikolata leke hastalığı yaygınlığını azaltmada *T. harzianum* izolatlarının hepsi hem *in vitro* hem de *in vivo* koşullarda ümit verici sonuçlar göstermiştir. Bununla birlikte izolatların etkinliği arasında varyasyonlar gözlenmiştir.

Anahtar kelimeler: Antagonistik değerlendirme, β-tubulin, EF1-α, ITS

EXTENDED ABSTRACT

Faba bean (*Vicia faba* L.) is among the important legume crop in Turkey. It is cultivated worldwide. The crop is hindered by several biotic and abiotic factors. Among these, a fungal disease caused by *Botrytis* species causes major crop losses by reducing production and productivities. Disease samples were collected from Adana province of Turkey and Haramaya provinces of Ethiopia in 2015/16–2016/17 and 2014/15, respectively, to determine the morphological, physiological and pathogenic variability of *B. cinerea* isolates as well as to identify the genetic variation among the population of *B. cinerea* and *B. fabae* using molecular method, and finally to evaluate the efficacy of isolates of *T. harzianum* against *B. cinerea* under *in vitro* and *in vivo* conditions.

Based on the results obtained from colony growth patterns, isolates of *B. cinerea* showed warty, radial, compact and powdery mycelia, and the colony colors were initially dirty white, grayish white and then turned gray to dark gray with age. It also produced black, hard, and irregular to flat shapes sclerotia 20 days after incubation at 17 ±2 °C. The maximum and minimum sclerotial sizes of Bc8 and Bc14 isolates were 3.0–6.0×2.0–4.5 and 1.5–4.0×1.3–3 mm (n=30), respectively. Moreover, the lowest (33) and highest (113) number of sclerotia per dish were counted on Bc2 and Bc9 isolates, respectively. Conidia developed in clusters, their color was pale brown, non-septate, shapes were elliptical to ovoid, and sizes were 7.0–14 × 6.0–11.5 and 7.0–12.5 × 5.5–9.5µm (n=50) on Bc6 and Bc8 isolates, respectively while conidiophores were septate and branched. Molecular identification of the *Botrytis* spp. based on combined DNA sequence data of two nuclear genes (ITS and β-tubulin) as well as morphological characteristics in this study showed that all isolates collected from the Adana province of Turkey were identified as *B. cinerea* while the other collected from Haramaya province of Ethiopia, were identified as *B. fabae*. *T harzianum* isolated from faba bean and field pea aerial parts were identified using a molecular approach (internal

transcribed spacer and translation elongation factor genes). As a result, all the isolates were identified as *T. harzianum*.

Results obtained from the effect of different temperatures on growth characteristics of *B. cinerea* and *T. harzianum* in the current study revealed that the colony diameter of *B. cinerea* isolates increased with each temperature up to 20 °C and declined thereafter. The fungus starts growing 7 and 3 days after inoculation at 5 and 10 °C. Nevertheless, the fungus completely covers the Petri plate within 6 days at 15 and 20 °C. Similarly, the optimum radial growth for all isolates was obtained at 20 °C and followed by 15 °C while no growths were observed at 0 and 35 °C. Moreover, the effect of temperature on the growth characteristics *T. harzianum* on PDA revealed that the highest colony diameter of the bio-agent fungus was recorded at 30 °C and followed by 25 and 20 °C, but the lowest was scored at 10 and 15 °C, and no growth was observed at 5 and 40 °C. Similarly, the highest RGR was calculated for all isolates at 30°C and followed by 25 °C. While the lowest RGR was scored at 10 and 35 °C, respectively.

Pathogenicity test using *B. cinerea* isolates on the faba bean leaflets incubated at 20±2 °C in the dark showed that symptom was developed after 72 hours, and the leaves were completely infected with the fungus 7 days after inoculation. Gradually, the pathogen started sporulation and developed sclerotia just after 12 days. Accordingly, the maximum lesion length and width were scored on Bc7 and Bc8, 7 days after inoculation. While the minimum was recorded on a Bc6 isolate. Similarly, the highest area under disease progress curves for lesion length was scored on Bc7 (104.2mm–days) and Bc8 (103.3mm–days), as well as the highest AUDPC (71.3 and 75.3mm-days) for lesion widths, were scored on these isolates, respectively, but the control leaflets inoculated with agar plugs, which was free of the fungus remained symptomless. Similar to detached leaf assay, Bc7 and Bc8 showed the highest disease severity of 62.9 and 67.9%, respectively on the whole plant test under glass-house conditions. In addition, the maximum progress of the disease symptoms over time (717.2 and 695.8%–days,

respectively) were computed on these isolates. However, control treatment showed the lowest disease severity (5.6%) and AUDPC (77.8%–days).

Antagonistic assessment of different isolates of *T. harzianum* against *B. cinerea* by dual culture assay showed variation in percentage inhibition of radial growth, inhibition zones and colony overgrowth day. The percentage inhibition of radial growth (PIRG) values ranging from 70.0 to 84.0% for a 1st method and 46.0 to 59.0% for the 2nd method. The highest percentage inhibition of radial growths (84.0 and 59.0%) was scored with the interaction of Bc8 × T2 and Bc7 × T2 for the method I and method II, respectively. Moreover, the highest inhibition zones (3.0 and 3.3 mm) were recorded in Bc4 × T1 and Bc4 × T4 for the 1st method and 2nd method, respectively. Antagonistic test on detached leaf showed that all isolates of *T. harzianum* reduced disease epidemics on detached faba bean leaves. The lowest disease severity (37.5%) and area under disease progress curve (65.6%-days) were calculated on T8 and T9 on the final day of disease assessment whereas leaves treated with *B. cinerea* alone exhibited the highest disease severity (68.8%) and AUDPC (131.3%-days).

The result of antagonistic assay against *B. cinerea* under glass-house conditions revealed that the highest disease severity was scored on control pots, which was inoculated with *B. cinerea* alone both in 2016 and 2017. While the lowest was scored on the pots inoculated with the interaction of *T harzianum* and *B. cinerea* isolates. The interaction of each Bc1 × T1 and Bc1 × T5 reduced disease severity by 66.7%, while Bc7 × T4 reduced the disease by 11.76% in 2016. In addition, the maximum AUDPC (1037.0 and 751.9%-days) were calculated on control (Bc3 × T0 and Bc4 × T0) pots in the first and second experiment, respectively. While the minimum (155.6%-days) was scored on a Bc1 × T1 pot of the first experiment. The study conducted under field conditions had also shown promising results in both 2017 and 2018 years. *T. harzianum* isolate, T6 reduced disease progress as compared with control plots.

The pathogenicity test experiment suggested that *B. cinerea* can infect faba bean where environmental conditions are conducive. Pathogenicity test using agar disk containing the fungal pathogen developed the symptoms faster than spore suspension inoculation. Bc7 and Bc8 were found to be the most virulent among screened *B. cinerea* isolates. Therefore, the present study pointed out that there was virulence variability among the isolates. On the basis of the results of this research, it can be concluded that effect of *T. harzianum* on faba bean chocolate spot caused by *B. cinerea* showed the promising result, even though the antagonistic ability of *T. harzianum* isolates varied. Growth and development of both *B. cinerea* and *T. harzianum* were highly influenced by temperature. Before deciding the application of *Trichoderma* spp. over any fungal disease control, knowing the temperature status of the study period needs to be explored.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	: Analysis of variance
AUDPC	: Area Under Progress Curve
Bc	: *Botrytis cinerea*
Bf	: *Botrytis fabae*
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
Bt2a	: Beta-tubulin forward primer
Bt2b	: Beta-tubulin reverse primer
CD	: Colony diameter
Cm	: Centimeter
CV	: Coefficient of variation
DAI	: Days after inoculation
DMRT	: Duncan multiple range test
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
FAO	: Food and Agriculture Organization
G	: Gram
h	: Hour
IRG	: Inhibition of radial growth
ITS4	: Internal transcribed spacer reverse primer
ITS5	: Internal transcribed spacer forward primer
L	: Litre
M	: Meter
Mg	: Miligram
$MgCl_2$	: Magnesium chloride
Ml	: Mililitre

n	: number
NaOCL	: Sodium hypochlorite
NCBI	: National Center for Biotechnology Information
P	: Probability value or calculated probability
PCR	: Polymerase Chain Reaction
PDA	: Potato Dextrose Agar
SD	: Standard deviation
SDS	: Sodium Dodecyl Sulfate
SE	: Standard error
SI	: Severity index
Sign.	: Significant
Str	: Streptomycin
T	*: Trichoderma*
TAE	: Tris-acetate-EDTA
TE	: Tris EDTA
Tet	: Tetracycline
µL	: Microlitre
°C	: Degree Celsius

1. INTRODUCTION

Faba bean (*Vicia faba* L.), also called Broad bean, Horsebean, Bakela in Ethiopia, Yeşil Bakla in Turkey, is an important cool-season legume crop grown worldwide (Graham and Vance, 2003). It is the fourth most produced grain legume worldwide next to chickpea, pea and lentil (Rebaa et al., 2017). Its center of origin is North Africa and South West Asia (Nedumaran et al., 2015), and it is the third most important crop grown under rain-fed and irrigated conditions in more than 50 countries. Seeds of faba bean have been used as a low-cost protein source for humans and animals (Maalouf et al., 2017). It is also grown for green pods and dried seeds (contain protein, 37.8%) which can substitute for animal protein in humans. Moreover, faba bean plays a significant role in the soil fertility enhancement by fixing nitrogen from the atmosphere through its symbiosis with *Rhizobium leguminosarum* in its root nodules and is an appropriate rotation with cereal crops (Ramadan and El-Kholy, 2014). It grows well in an optimum temperature ranging between 18 and 27 °C, and it needs soil with high contents of loamy and clay-loam with neutral to alkaline pH (Tefere, 2015).

Faba bean is cultivated nearly on 2.4 million hectares of lands and production is around 4.5 million tons worldwide according to Faostat (2018). From these, China is the leading producer with 1.6 million tons followed by Ethiopia (878,010 tons) and Australia (423,527 tons); while Turkey ranks 23rd with 14,489 tons (Faostat, 2018) (Table 1.1). Faba bean is one of the major food legume crops in the Mediterranean region of Turkey (Karaköy et al., 2014) (Table 1.2 and Figure 1.1). In this country, It occupies the 4th rank among legume crops in terms of production, after chickpea, lentil and dry bean (Tufan and Erdoğan, 2017), and is consumed as a fresh vegetable as well as in canning and freezing industries. It is mainly grown in the Mediterranean (Adana, Antalya, Hatay and Mersin etc.) followed by Marmara, Aegean region, Anatolia, Black Sea, and Central East Anatolia Region (Balkaya and Karağaç, 2013).

Table 1.1. The major faba bean producing countries in the world with respect to production and area coverage

Production			Harvested area (Acreage)		
Rank	**Countries**	**Tons**	**Rank**	**Countries**	**Hectare**
1	China	1608903	1	China	806858
2	**Ethiopia**	**878010**	**2**	**Ethiopia**	**427697**
3	Australia	423527	3	Australia	350457
4	UK	288955	4	Morocco	82774
5	France	198246	5	France	78000
6	Germany	153700	**26**	**Turkey**	**5292**
7	Sudan	121412			
8	Egypt	119104			
9	Italy	100013			
23	**Turkey**	**14489**			

Source: Faostat, 2018

Table 1.2. The leading faba bean producing provinces in Turkey with respect to production and area coverage

Production			Harvested area (Acreage)		
Rank	**Province**	**Tones**	**Rank**	**Province**	**Hectare**
1	Antalya	7323	1	Mersin	694.7
2	Mersin	6769	2	Antalya	692
3	Balıkesir	4211	3	İzmir	410.9
4	İzmir	3852	4	Balıkesir	398.5
5	**Adana**	**2085**	5	Muğla	212.6
6	Bursa	1627	6	Manisa	191.7
7	Aydın	1557	7	Çanakkale	176.8
8	Çanakkale	1493	8	**Adana**	**157.8**
9	Manisa	1283	9	Bursa	147.8
10	Muğla	1247	10	Aydın	124.4

Source: TurkStat, 2017

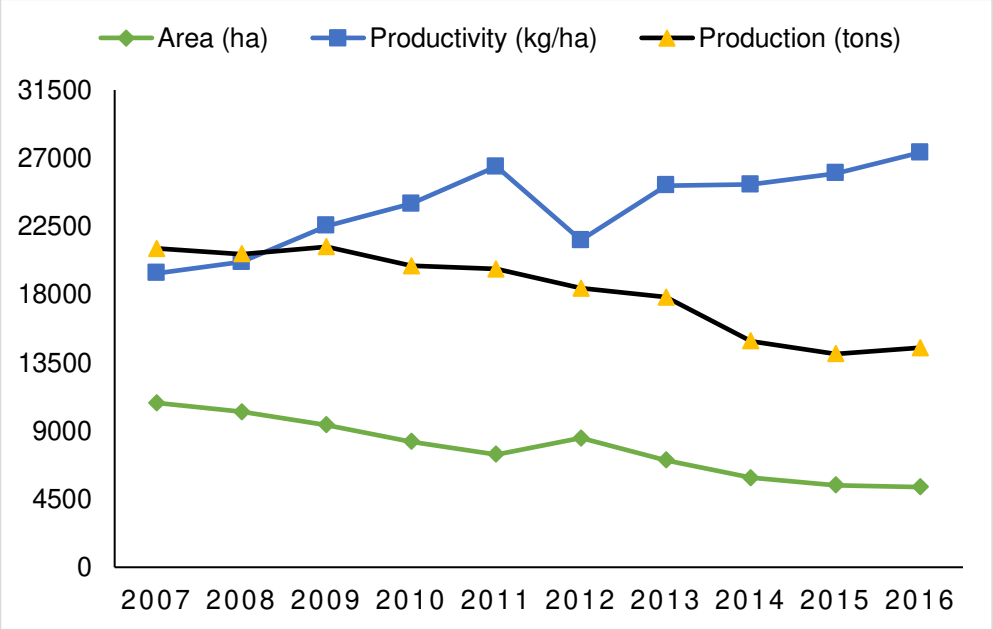

Figure 1.1. The trend of faba bean area coverage, production and productivity in Turkey (Faostat, 2018)

Although faba bean is one of the most important legumes in Turkey, the yields are constrained by fungal diseases like Ascochyta blight caused by *Ascochyta fabae*, rust (*Uromyces faba*), chocolate spot (*Botrytis* spp.), *Alternaria alternata*, root rots and damping off (caused by *Rhizoctonia* and *Pythium* spp.) (CAB International, 1981; Kitiki and Kutlu, 1991; Kayım et al., 2018). *Aphis fabae* and *Orobanche crenata* are also the major limiting factors in faba bean production; and abiotic factors, such as high temperature, excessive moisture, drought, and salinity and nutrient deficiency also play a role (Terefe, 2015).

Among these, chocolate spot caused by *Botrytis fabae* Sard. (teleomorph: *Botryotinia fabae* J. Y. Lu and T. H. Wu) (Wu and Lu, 1991) and *Botrytis cinerea* Pers. Ex. Fr (teleomorph: *Botryotinia fuckeliana*) has been recorded as one of the most detrimental foliar diseases of this legume crop since 1849 (Harrison, 1988; Sahile, 2008). These fungi are known by its asexual form, and the sexual phase is rarely observed (Sahile, 2008). Sometimes both species are considered equally

responsible for disease development, even though *B. fabae* is more aggressive than *B. cinerea* in causing disease (Harrison, 1988; Sahile, 2008). Because *B. fabae* has the ability to metabolize the host's phytoalexins faster than the later fungus. However, under conducive environmental conditions, *B. cinerea* can be induced to the aggressive phase (Hargreaves et al., 1977). In addition, the conidial size of *B. cinerea* is much smaller than *B. fabae*. In addition, it produces few numbers and larger sclerotia than *B. fabae* (Davidson et al., 2007). Recently, Zhang et al. (2010) reported a new species of *Botrytis fabiopsis* causing chocolate spot of faba bean in central China. They have threatened the faba bean crop in the major faba bean growing regions of the world (Rahman et al., 2002). Chocolate spot symptom on leaves and flowers are very common, yet under favorable conditions, the disease may spread to the stems and pods, as a result, they can cause pods rot, blossom blight and leaf blight. Discrete reddish-brown spots to non-aggressive phase and an aggressive phase occur when spots darken in color and coalesce to form larger gray-brown target spots that may eventually cover the entire plant (Zhang et al., 2010; Davidson and Lindbeck, 2016). Under humid and cool weather conditions, the chocolate spot can severely devastate faba bean as well as limit growth and seed development. In China, the yield loss caused by this disease can reach 50-100% (Zheng et al., 2008). Moreover, yield losses of up to 90% in Australia, 61% in Ethiopia, and 59% in the UK (Davidson et al., 2007) were reported. So far, there have been no quantified data on yield loss caused by these fungi in Turkey.

Outside the growing season, these fungi can survive as sclerotia in soil or on crop debris, in or on infected seed or on the self-sown volunteer plants. Infection is spread when the wind carries the airborne fungal spores from one area to another within 4-5 days of initial infection. Spores can then be formed on infected tissue and initiate a secondary spread of the disease within and between crops (Davidson and Lindbeck, 2016).

Accurate identification of the fungal pathogens is a precondition for population genetics and ecological studies as well as for effective management of

the diseases they cause (Njambere et al., 2010), and It is also important to understand the evolution of morphological characteristics (Raja et al., 2017).

Morphological characteristics, such as mycelia, sclerotia, and conidia are useful in the identification of certain fungal species. Nevertheless, the majority of the species are phenotypically similar. Indeed, a key to all recognized species is not available. Rather than one morphological trait, species may be identified by phylogenetic analysis of variable nucleic acid sequences. Morphological characters, together with DNA sequence data of multiple protein-coding genes can be used clearly to identify *Botrytis* species and open the way to a better understanding of the genetic diversity within the genus and species (Staats et al., 2005). The classical method used to differentiate *Botrytis* species are based on morphological characterization of their conidia and teleomorph as well as colony appearance and symptoms (Corbiere and Bouznad, 1998). Combining neutral molecular markers with pathogenicity assays represents a potentially useful method of investigating spatial and host differentiation in pathogen populations (Abang et al., 2006). Genetic information required to design and deploy *Botrytis* disease management tools can be obtained using a wide array of molecular techniques (Mirzaei et al., 2007), but the utility of these techniques for faba bean chocolate spot agent population genetic analysis has not been evaluated (Sahile et al., 2012). In addition, classical studies based on the analysis of macroscopic and microscopic features, and their characteristics may change considerably based on the conditions and the environment they are exposed; and the use of these methods for classifying fungi at the higher taxonomic levels (family, order, class) could not always perform well at taxonomic levels because morphological characters can be problematic even for trained peoples in this field, as they may not always provide accurate groupings within an evolutionary framework, mainly at the lower taxonomic levels (species and genus). They can be misleading due to cryptic speciation, convergent evolution, and hybridization (Raja et al., 2017). In addition, many fungal species are similar phenotypically, and accurate species identification

can be difficult (Njambere et al., 2010). According to the latest International Code of Nomenclature for fungi, this practice is no longer acceptable.

Molecular methods are quicker and sensitive. Moreover, they are used for the specific detection of the fungal species *(*Zarrin et al., 2016). As a consequence, DNA sequencing methods have been developed for the quick identification of fungi (Njambere et al., 2010; Raja et al., 2017). The development of specific primers for PCR amplification (White et al., 1990) has helped these studies and the identification of fungal pathogens. Universal primers, such as internal transcribed spacer (ITS) region of nuclear ribosomal DNA, has been employed for molecular identification of fungi at lower taxonomic levels because the ITS exhibits high variation, evolves the fastest and conserved within species. Besides, it is easier in amplification, and appropriate large barcode gap makes ITS gene the most useful gene region for fungi identification (Njambere et al., 2010; Raja et al., 2017). The use of an internal transcribed spacer (ITS) alone for identification of some fungi like *Trichoderma* spp. may not be sufficient. Therefore, single-copy protein-coding gene, such as translation elongation factor has been used worldwide for identification of fungi at the species level (Raja et al., 2017).

A detached leaflet test has been widely used for resistance screenings and for analyzing components of resistance or assaying different factors affecting the response of faba bean to *B. fabae* (Villegas-Fernandez et al., 2012). Furthermore, the whole plant test allows the study of the plant as a whole organism interacting with the pathogen instead of being limited to specific areas (Villegas-Fernandez et al., 2012).

Growers frequently spray fungicides for the management of these diseases. However, fungicides are hazardous to human health, it is costly and reducing the crop's profitability (Fernandez-Aparicio et al., 2011). In addition, repeated applications of chemical fungicides have a negative impact and their residues cause a lot of problems such as environmental pollution and resistance in disease-causing organisms to fungicides and can cause a phytotoxic effect on beneficial plants (El-

Gali, 2015). For instance, the development of resistance in *B. cinerea* and *B. fabae* against fungicides has been reported (Sahile et al., 2011). Breeding of resistant cultivars is a priority to control the disease, but no completely resistant cultivars have been developed so far (Fernandez-Aparicio et al., 2011). Management of chocolate spot caused by *B. fabae* is possible through intercropping of faba bean with cereals and legumes like vetch (*Vicia sativa* L.) and field pea (*Pisum sativum* L.) (Fernandez-Aparicio et al., 2011), because *B. fabae* has a narrow host range, but this is not a case for *B. cinerea*, which attacks more than 200 dicotyledon crops, including *V. faba* and others worldwide (Williamson et al., 2007).

Therefore, to overcome these difficulties, it is better to apply alternatively safe and efficient methods against these fungal diseases. Aerial parts of a plant also called phylloplane or the phyllosphere, provide a habitat for several of epiphytic microorganisms, the majority of which inhibit the physiological activity of pathogens (Jackson, 1993).

Trichoderma spp. are free-living fungi that are highly interactive in the root, soil, and aerial environments and commercially marketed as bio-pesticides, bio-fertilizers and soil amendments (Abd El-Rahman and Mohamed, 2014). The use of *Trichoderma* fungi in agriculture can provide numerous advantages; (I) control of plant pathogens by antibiosis production, parasitism or competition (II) colonization of rhizosphere of a plant, and (II) promoting plant growth, stimulation of plant resistance and immunity against pathogens (Harman et al., 2004), promote plant vigor and growth as well as to impart tolerance to abiotic stresses (Mukherjee et al., 2013). Moreover, *Trichoderma* spp. are broad spectrum, adaptive to different environmental conditions, and grow faster than plant pathogens (Wu et al., 2017). They are best known for their ability to produce enzymes that degrade cellulose and chitin and also produce other useful enzymes (Motlagh and Samimi, 2013). They are the most common biocontrol agents of plant pathogenic fungi that caused soil-borne, airborne and post-harvest diseases in several crops (El-Gali et al., 2015). Several studies have investigated the role of *Trichoderma* as bio-control

agents against diseases of leaves spots and plant growth enhancement (Prasad et al., 2013).

The use of *Trichoderma* spp. as a biological control is an important tactic for plant disease management caused by fungi in general and for faba bean chocolate spot disease management in particular. So far, studies on evaluations of *Trichoderma* spp. on various soil-borne pathogens have been conducted using commercial and by isolating from the soil in Turkey. Among these, commercial KRL AG2 of *T. harzianum* at different doses were evaluated against root rot disease (*Rhizoctonia solani, Fusarium solani*) of cucumber (Yücel et al., 2008); isolation, production, and characterization of an extracellular lipase of *T. harzianum* isolated from soil by Ulker et al. (2011); biological control of chestnut blight using isolates of *Cryphonectria parasitic*, *Trichoderma* sp., *Penicillium* sp., and *Bacillus* sp. (Akilli et al., 2011); isolation of *Trichoderma* sp. from soil and determination of their antifungal, biochemical and physiological features by Küçük (2003); the adaptation of *T. harzianum* T22 race to different pH and salt concentrations and its user in biological control of *F. oxysporum* f.sp. radicis lycopersici on tomato (Yiğit, 2011). Fungal antagonists have not been explored for the biological management of chocolate spot disease of faba bean. *Trichoderma* species can inhibit the radial growth of *B. fabae.* Still repeated evaluation of *Trichoderma* spp. using different isolates under laboratory, greenhouse and field conditions are very important (Sahile et al., 2011). Recently, *T. harzianum* Rifai strain KRL-AGZ and *Bacillus subtilis* QST 713 strain was evaluated for their effects on *B. cinerea* (Pers) in lettuce, significantly reduced disease epidemics (Polat, 2010).

1.1. The Problem of the Statement, Justification and Objectives

Faba bean chocolate spot caused by *B. cinerea* and *B. fabe* is distributed throughout the world and causing economic losses. It can limit photosynthetic activity and reducing faba bean production and productivity (Torres et al., 2006).

Yield loss because of this disease may reach 80% among the susceptible cultivars, and 35% among the resistant cultivars.

Despite the importance of faba bean chocolate spot pathogen, there have been no studies in the Eastern Mediterranean Region of Turkey, particularly regarding its morphological and molecular characterization, which are essential for successful disease management. Hence, the present dissertation was initiated with the following specific objectives: (1) to investigate the morphological and pathogenic variability of *B. cinerea* isolates, (2) to identify the genetic diversity between *B. cinerea* and *B. fabae* isolates and (3) to assess the efficacy of *Trichoderma harzianum* against faba bean chocolate spot both under *in vitro* and *in vivo* conditions.

2. LITERATURE REVIEW

2.1. Origin and Economic Importance of Faba Bean

According to Cubero (1974) and Duc (1997), faba bean is one of the earliest crops to be domesticated and the geographic origin of faba bean (*Vicia faba* L.) is accepted as the near East. Muehlbauer (1993) stated that faba bean is a grain legume grown worldwide as a source of protein for both human food and animal feed. In some countries, including Turkey, the faba bean pods and seeds are consumed directly as a green vegetable. Maxted and Kell (2009) suggested that the Mediterranean basin as the most important center of diversity for *Vicia faba.* According to Njambere et al. (2010), faba bean, chickpea, lentil and pea, are important cool season grain legumes crops worldwide. Jensen et al. (2010) stated that in addition to protein, cropping faba bean benefits the ecosystem with renewable inputs of nitrogen (N) available for the next crops in the rotation and soil from biological N_2 fixation and diversification of cropping systems. According to Paull et al. (2011), faba bean was introduced to South America by the Spanish and was established as a crop in Australia in the 1980s.

Beyene (2014) mentioned that China, Afghanistan, and Ethiopia have also been reported as secondary centers of diversity for this crop. According to Terefe (2015), the amount of atmospheric nitrogen fixed through symbiosis in faba bean exceeds that from peas. Yields of cereal crops following faba bean are therefore improved, thus reducing the need for artificial nitrogen fertilizer for subsistence farmers. Mitiku (2017) stated this crop belongs to tribe Vicieae. Herbs with pinnate leaves and grows upright, ranging from 1-1.5 meters tall. It is an annual legume with one or more strong, hollow and erect stems. It has a strong taproot, compound leaves, and large, white flowers with dark purple marking. A flower cluster may produce 1-4 pods. The pods are large (up to 10 cm long and 1-2 cm wide) and green, turning dark at maturity.

2.2. Biotic Factors Constraint Faba Bean Production

According to Yu (1979), out of a total of 102 faba bean diseases caused by fungi, bacteria or viruses, rust *(Uromyces fabae*), chocolate spot (*Botrytis fabae*), ascochyta blight (*Ascochyta fabae*), zonate spot *(Cercospora zonata* Wint.) and root rot *(Fusarium solari* Mart.) are the most common ones and cause devastation on yield and yield components.

Hanounik and Robertson (1989) suggested that faba bean is adversely infected by numerous fungal diseases. The majors are rust (*Uromyces fabae*), black root rot (*Fusarium* spp.), downy mildew (*Peronospora viciae*) and ascochyta blight (*Ascochyta fabae* Speg). According to Duc (1997), there is a reduction in the cultivated area and the productivity of faba bean in many countries. Several other adverse factors have been reported to cause this decline. Among insect pests, aphids (*Aphis fabae* Scopoli, *Aphis craccivora*, and *Acyrthosiphon pisum*) and potato leafhoppers (*Empoasca fabae* Harris) cause serious damage to the crop in countries like Sudan and Egypt. Moreover, *Helicoverpa armigera* (pod borer), *Sitona lineatus* (the leaf weevil), *Sitona amurensis* (root nodule weevil), *Agrotis* spp. (Cutworms), *Liriomyza congesta* (leaf miner), *Spodoptera exigua* (lesser armyworm), *Bruchus* and *Callosobruchus* spp. (Bruchids) are major post-harvest pests in countries like Ethiopia.

According to Muehlbauer and Tullu (1997); Zhang et al. (2010), faba bean is adversely infected by chocolate spot (*Botrytis fabae*, *Botrytis cinerea*, and *Botrytis fabiopsis*). This disease results up to 90% yield loss in faba bean. Gressel et al. (2004) mentioned that the crop is also damaged by parasitic weed like *Orobanche crenata* which is widespread in the Mediterranean basin, especially in northern Africa, Asia and southern and eastern Europe, attacking dicotyledonous crops, and losses of 50 to 80% have been reported in faba bean fields. There is no practical control measure applied against it. On the other hand, root-lesion nematodes (*Pratylenchus* spp.), stem nematodes (*Ditylenchus dipsaci*) and root-

knot nematodes (*Meloidogyne* spp.) also infect this legume crop (http://uses.plantnet-project.org/en/Vicia_faba_(PROTA)).

In addition to the above factors, O'Sullivan and Angra (2016) stated that abiotic stresses like heat and drought also play an important role in threatening faba bean production and productivity in the world because of the sensitivity of flower fertility to these stresses. According to a report from Australia by GRDC (2017), root rots and damping off are mainly caused by *Fusarium*, *Rhizoctonia*, *Pythium* spp. and *Phoma medicaginis* occasionally be a serious disease when soils are wet for prolonged periods. Faba beans are also infected by more than 50 viruses worldwide, and the number continues to increase. Among these; Bean leaf roll virus (BLRV), Beet western yellow virus (BWYV), Soybean dwarf virus (SBDV) (syn. Subterranean clover), red leaf virus (SCRLV), Subterranean clover stunt virus (SCSV), Clover yellow vein virus (ClYVV), Bean yellow mosaic virus (BYMV) and Broad bean stain virus (BBSV) are very common worldwide.

2.2.1. Occurrence and Economic Importance of Faba Bean Chocolate Spot

According to Wilson (1937), faba bean chocolate spot was first described by Berkeley near the Northampton in 1849. Sardina had also described *Botrytis fabae* and *Botrytis cinerea* Pers. as one of the most common diseases on *Vicia faba* in 1931. Since then it has caused devastation on broad bean worldwide. According to Harrison (1988), several cycles of infection may take place in a short period of time, particularly when mild temperatures and high relative humidity occur, giving rise to extended necrosis that can frequently cause defoliation and even death of whole plants. According to Muehlbauer (1993), *Botrytis* gray mold of faba bean can cause yield reductions in the humid period.

As reported by Yu and Sutton (1997), *B. cinerea* is characterized by abundant hyaline conidia (asexual spores) borne on gray, branching treelike conidiophores. The fungus also produces highly resistant sclerotia as survival structures in older cultures. It overwinters as sclerotia or intact mycelia,

both of which germinate in spring to produce conidiophores. Conidia can be dispersed by wind and by rainwater, cause new infections. According to Torres et al. (2006), the chocolate spot is one of the economically important diseases that damage the foliage, limiting photosynthetic activity and reducing faba bean production. Stoddard et al. (2010) had also stated as important disease of faba bean throughout the world. According to Zhang et al. (2010) and Cheesman (2014), when relative humidity is more than 90% and air temperature ranges between 15-22 °C, this disease is prevalent causes devastation and leads to yield losses up to 100%.

Zhang et al. (2010) revealed that in faba bean, three species have been shown to cause disease: *B. cinerea*, *B. fabae* and most recently *B. fabiopsis*. El-Hendawy et al. (2010) stated that yield losses caused by this disease can reach up to 60-80% and 34% on susceptible, and tolerant cultivars, respectively in African regions like in the Maghreb Nile Delta. According to Villegas-Fernandez et al. (2012), only low levels of resistance are available in faba bean cultivars against the disease.

2.2.2. Symptoms

According to Harrison (1984), the percentage of lesions produced both by *B. cinerea* and *B. fabae* increased as the season progressed. Infection levels of *B. cinerea* increased at a lower but steadier rate over the whole growing season and that of *B. fabae* tended to increase rapidly during midsummer. Lesions caused by *B. cinerea* appeared similar to those caused by *B. fabae* under various environmental conditions.

Honounik and Bisri (1991) revealed that faba bean damage by chocolate spot is related to plant age at the time of infection. Moreover, matured faba beans are more susceptible than young seedlings. Another study done by Honounik and Bisri (1991) showed that early chocolate spot symptoms occur mainly on leaves. Nevertheless, pods, stems, and flowers may also become infected. Non-aggressive and aggressive stages of the disease can also be observed according to the above author. The non-aggressive stage is when the small circular and discrete reddish

spot with darker margins occur, and aggressive stage when the small spots merge and coalesce to form irregular larger dark-brown lesions involving the whole leaf surface.

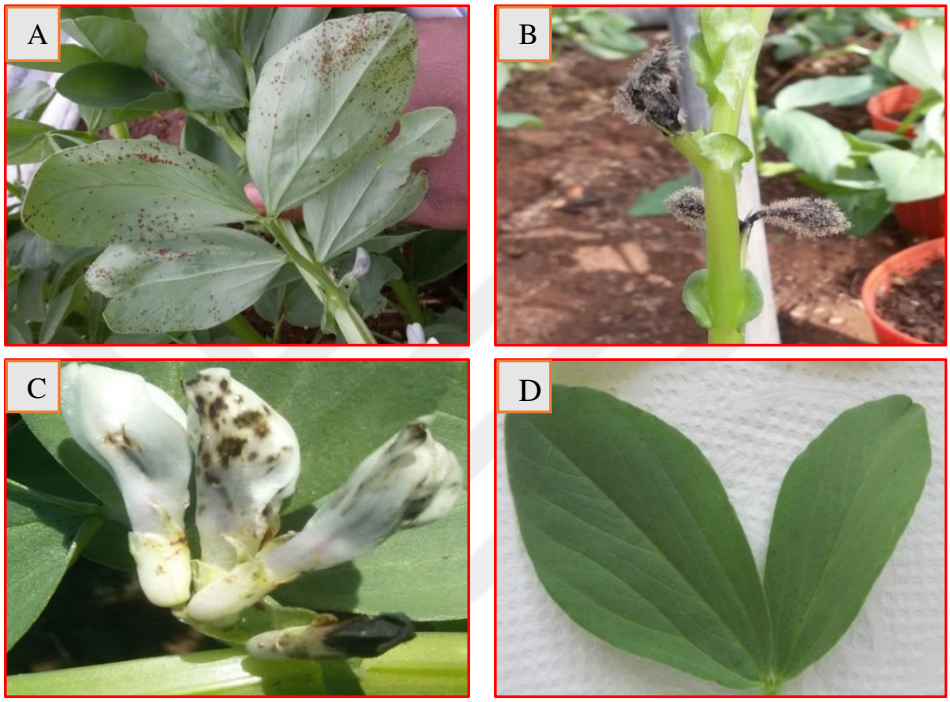

Figure 2.1. Chocolate spot caused by *B. cinerea* on faba bean; reddish brown spots on leaves (A), sporulation on leaves (B), symptom on blossoms (C), and symptomless (D)

According to Donna (2016), the symptoms of the disease reflect the name with small chocolate spots on the leaves appearing first. When symptoms first appear, it looks like someone took a paintbrush and splattered paint on the leaves, giving the lower leaves a peppered appearance. The chocolate colored lesions start small but can start to expand if moisture is available under warmer temperatures, eventually merging so that the whole leaf turns brown. After two or three weeks, the larger lesions will turn gray, looking more similar to *Botrytis* or gray mold in other crops. The disease does produce small sclerotia bodies similar to *Sclerotinia*

sclerotium (white mold). These sclerotia bodies can be found inside the stems of badly diseased plants.

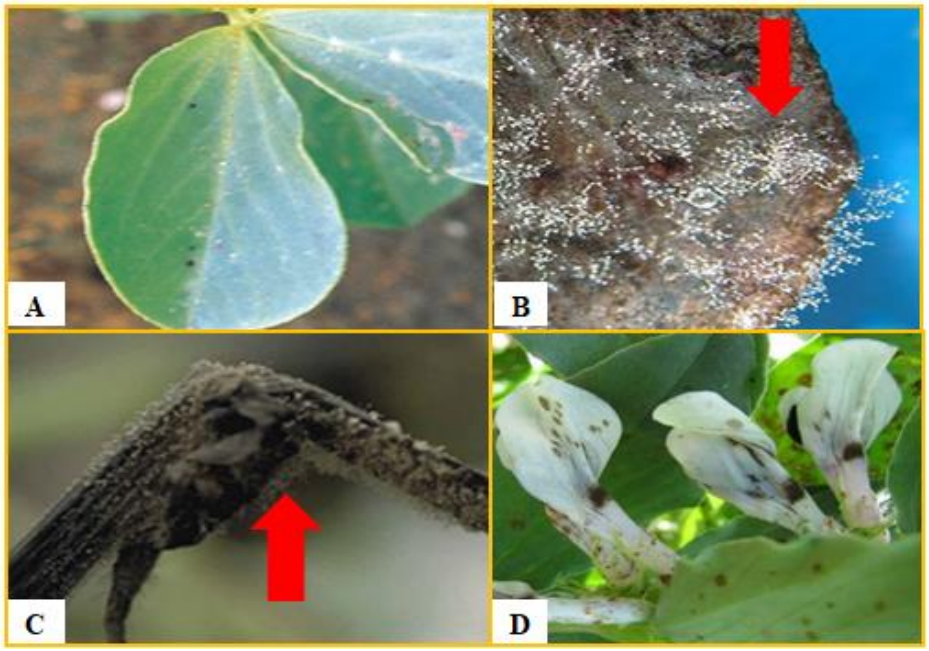

Figure 2.2. Chocolate spot caused by *B. fabae* on faba bean; infections of a chocolate spot in beans start as small brown spots (A) (photo by Grain Legume Handbook); *chocolate* spot (*Botrytis fabae)* lesion showing lesion expansion and sporulation after a few days in a humid chamber (B) *(*Photo: Joop Van Leur, NSWDPI); chocolate spot on stems (C) *(*Photo: Grain Legume Handbook); D Chocolate spot on flowers by SARDI (D) (GRDC, 2017).

2.2.3. Disease Cycle and Host Specialization

According to Staats et al. (2005), chocolate spot caused by *B. fabae* can infect all Fabaceae family like soybean (*Glycine max*), lentil (*Lens culinaris* subsp. culinaris), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and faba bean (*Vicia faba*). While the *B. cinerea* infecting more than 200 dicotyledon plants including faba beans.

As reported by GRDC (2017), the fungus may either survive as sclerotia in the soil or on crop debris, on infected seed, or on the self-sown volunteer plants (Figure 2.3). In new faba bean growing areas, the disease often becomes established by sowing infected seeds. In subsequent years, the initial infection usually occurs when spores formed on old bean trash are carried by the wind into new crops. These spores may move long distances. Once the disease becomes established, it rapidly spreads within a crop and within 4-5 days of infection, spores can be formed on infected tissue and initiate the secondary spread of the disease. As described by Dean et al. (2012), the sexual fruiting bodies of *Botrytis cinerea* are showing in Figure 2.4. Donna (2016) had also mentioned that the disease typically develops later in the season during flowering and after canopy closure. The fungus is more aggressive under warm (15–25°C) humid conditions (>70% RH) that extend from 4–5 days and can spread rapidly within a crop.

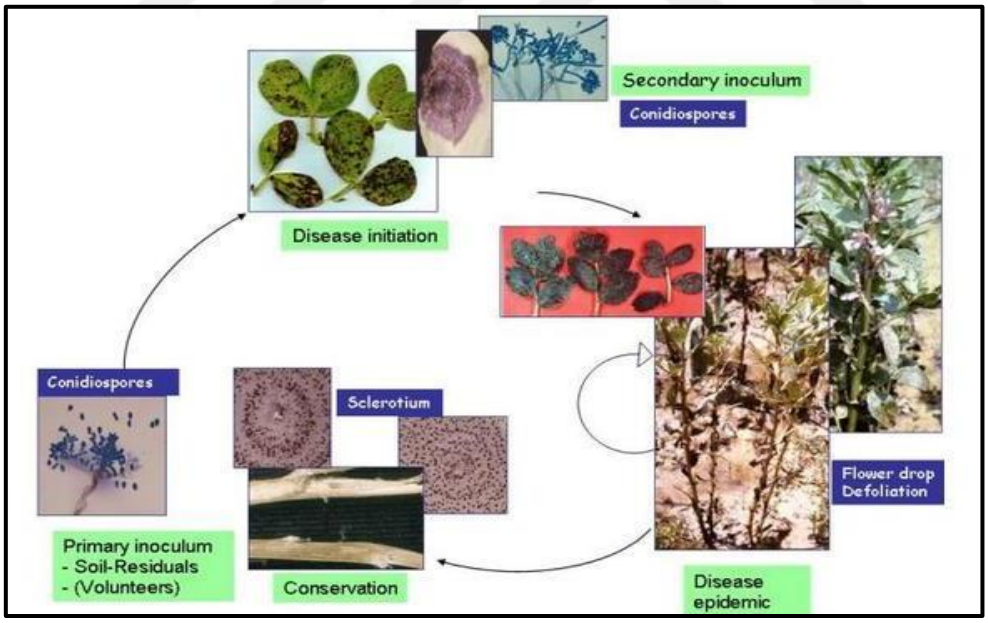

Figure 2.3. The life cycle of chocolate spot causal fungi on faba bean (Bernard TIVOLI, INRA).

Figure 2.4. Sexual fruiting bodies (apothecia) of the teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel

2.2.4. Taxonomy

According to CAB (1981), *B. cinerea* is usually referred to by its anamorph name because the sexual phase is rarely observed. The teleomorph is an ascomycete, *Botryotinia fuckeliana*, also known as *Botryotinia cinerea*. *B. cinerea* is classified under the kingdom: fungi, phylum: Ascomycota, class: leotiomycetes, order: Helotiales, family: Sclerotiniaceae, genus: *Botrytis*. Assadollahi (2013) mentioned that the name of *Botrytis cinerea* is derived from the Latin word "grapes like ashes"; although poetic, the "grapes" ("botrytis" from Ancient Greek botrys meaning "grapes" plus the Neolatin suffix–it is for disease) refers to the bunching of the fungal spores on their conidiophores, and "ashes" just refers to the greyish color of the spores en masse.

2.2.5. Morphological and Molecular Characterization of Chocolate Spot and *Trichoderma* Species

A study conducted by Xun-Yi (1989) showed that there was morphological variation among *B. fabae* isolates; accordingly, some of them produce few sclerotia

with excessive growth of mycelium, while the other produce abundant sclerotia with minimal growth of mycelium, and the others also show moderate production of sclerotia and mycelium. As described by Kindermann et al. (1998), for the first time, phylogenetic analysis of the whole genus of *Trichoderma* using the ITS1 region of rDNA was done.

According to Ospina-Giraldo et al. (1998), the ITS gene region is among the most widely sequenced DNA regions in fungi. It has been used for molecular study at lower taxonomic levels of fungi such as species. A study conducted by Choi et al. (1999) stated that the method of identifying fungi does not rely solely on morphological features for identification. The use of fungal cultures, obtained from single spore isolations is fundamental to the identification of many fungi.

A study conducted by Kwon and Park (2003) showed that the *B. cinerea* occurred severely on *Vicia faba* grew in farmer`s fields located in Changseon-Myon, Namhae-gun, Gyeongnam province, Korea. Symptoms first noticed on leaves showed discolored to gray or darker from the tip, and eventually died. Conidia of the fungus were single-celled, gray or hyaline, mostly ovoid or ellipsoid and sized 6–22 × 4–16 μm. Conidiophores thickness was produced on PDA with 15–37 μm in width. The sclerotia were produced on PDA and optimum temperature for sclerotial formation is 20 °C. However, the pathogenic variability among the isolates was not investigated.

Sadfi-Zouaoui et al. (2009) identified different *Trichoderma* strains from a different ecosystem of Tunisia using the translation elongation factor 1 (tef1) and ITS1 and ITS2) of the rDNA genes. Accordingly, they were identified as *T. hamatum* and *T. atroviride* in cultivated lands; *T. longibrachiatum* and *T. harzianum* clade VI were in forest soils, and *T. hamatum* and *T. harzianum* clade II were present in oasis soils.

According to a study carried out by Zhang et al. (2010) in China, among faba bean chocolate spot causal fungi, *B. cinerea* was the most distributed with

53.8%, followed by *Botrytis fabiopsis* (36.4%) and *B. fabae* (9.8 %). *B. cinerea* was identified in more than 49 countries. In addition, morphological features of this fungus were characterized by the formation of gray to white, fluffy and villiform aerial mycelia and gray to black sclerotia, while *B. fabae* strains produced abundant black sclerotia and glistening white ropy mycelia. On the other hand, *B. fabiopsis* formed gray to white aerial mycelia, which resemble mycelia of *B. cinerea.* It's also known for the formation of few and gray to black sclerotia in concentric rings. Moreover, the above authors had also confirmed that mycelial agar plugs of *B. cinerea* were more aggressive than *B. fabae* and *B. fabiopsis* in causing chocolate spot disease in faba bean. On the other hand, conidia of *B. cinerea* was less virulent than *B. fabiopsis and B. fabae* in causing the symptoms.

As reported by Zhang et al. (2010); Walker et al. (2011); Li et al. (2012); Zhou et al. (2014), the cultural and morphological characteristics, as well as molecular characterization using RPB2, G3PDH and HSP60 primers, were very important in the delimitation of *Botrytis* species.

Hoyos-Carvajal and Bissett (2011) reported several gene regions for delimiting *Trichoderma* species, such as RNA polymerase II subunit B2 (RPB2), calmodulin 1 (CALM1), chitinase 18-5 (ECH42) actin, β-tubulin2 (TUBB2), translation elongation factor 1-α (TEF), ATP citrate lyase subunit A (ACLA) and LAS1 nuclear protein, which can provide informative loci for multi-gene studies. According to these authors, *Trichoderma* spp. could respond to different cultural media, media like malt extract agar is crucial for the observation of complex branching conidiophores production of conidia. While a medium like a potato dextrose agar is appropriate for getting maximum mycelium to extract DNA and observing the color (pigment) production. Similarly, Behr et al. (2013) reported that specific primers, β-tubulin1 (Bt1) and β-tubulin 2 (Bt2) targeting the β-tubulin gene, and ITS1-ITS4 and ITSU5-ITSR2 targeting the ITS region were employed for the identification of *B. cinerea*-related species.

A study conducted by Abd-Rabh et al. (2013) stated that the chocolate spot caused by *B. cinerea* and *B. fabae* showed a different linear growth rate on three media (faba bean seed agar medium, Potato dextrose agar and faba bean leaf agar). *B. cinerea* isolates grew faster on a PDA than *B. fabae*, while the maximum sporulation was produced on faba bean seed agar. Moreover, abundantly sclerotia were produced on PDA and followed by faba bean seed agar although the size and number of sclerotia were varied among the isolates.

Fan et al. (2015) designed the following species-specific PCR primers, Bc-f/Bc-r for *B. cinerea* and Bfab-f/Bfab-r for *B. fabiopsis*, and Bfa-f/Bfa-r for *B. fabae* to detect and differentiate *Botrytis* species causing faba bean chocolate spot.

In India, Savitha and Sriram (2015) identified 10 *Trichoderma harzianum,* three *Trichoderma asperellim* and two *Trichoderma virens* using rDNA region with primers ITS1 and ITS4 (products with 600 bp) and translation elongation factor, *tef1-α* region with 300 bp. DNA sequencing data of these regions were determined in distinguishing among isolates of *Trichoderma* species. Braithwaite et al. (2016) used approximately 900 bp tef71f and tef997R primer pair to amplify *tef1-α* gene. As a result, 320 *Trichoderma* species were identified in New Zealand.

Chennappa et al. (2017) extracted genomic DNA from *Trichoderma* isolates by using the CTAB method and ITS1 and ITS5 primers were employed for the amplification and confirmed that the amplified DNA length was in the range of 600-650 bp. Accordingly, four of them were identified as *T. virens,* six were *T. harzianum*, and two were *T. piluliferum, T. asperellum* as well as three were identified as *T. viride.*

Aktaruzzaman et al. (2017a) described morphological characteristics of *Botrytis cinerea* isolated from the green bean in Korea; its colony was white to gray or dark gray, conidial shape and color were ellipsoidal to ovoid and dark brown, respectively, and sizes were 5.1–8.5 × 5.2–9.8 µm. Sclerotia shape and color of the fungus were flat with irregular and black, respectively, as well as sizes

were 1.2–4.3 × 1.1–3.5 µm. The authors had also identified fungus using a molecular approach. For this study, universal primer, an internal transcribed spacer (ITS1 and ITS2) and 5.8S ribosomal DNA as well as nuclear protein-coding genes, DNA-dependent RNA polymerase subunit II gene (RPB2), glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) and heat-shock protein 60 gene (HSP60) were employed.

2.2.6. Effect of Temperature on Radial Growth of *B. cinerea* and *Trichoderma* spp.

Brooks and Cooley (1917) found that an optimum growth rate of *B. cinerea* is at 20-22 °C and declined noticeably above 25 °C. However, few authors had also described that an optimum temperature for mycelial growth of *Botrytis cinerea* was 24-28 °C on potato sucrose agar, with a maximum of 35 °C and a minimum of 0 °C. Similar results were observed by Morotchkovski and Vitas (1939) that the optimum temperature range of sclerotial production in *B. cinerea* about 11-13 °C. However, the fungus can sporulate at a temperature range of 12-22 °C and can form appressorium at 27-28 °C.

According to Kublitskaya and Ryabtseva (1972), the temperature requirement for favoring sclerotial production in *B. cinerea* commonly hinder mycelial production and vice versa. Besides, Kochenko (1972) described that *B. cinerea* produced sclerotial at the optimum temperature between 22 °C and 24 °C. A study conducted by Garrido and Sônego (2005) showed that the growth and development of *Botrytis* spp. were favored by temperatures ranging from 18 to 23°C.

Similarly, Hajieghrari et al. (2008) reported that the highest mycelial growth of *T. harzianum* and *T. hamatum* were recorded at a temperature of 25 ºC whereas the mycelial growth of *T. virens* was high at 30 ºC. A study conducted by Hosen et al. (2010) stated that the optimum pH and temperature for the *B. cinerea* mycelial radial growth were 4.5 and 20°C, respectively. The growth rate increased

up to the temperature of 20°C then it reduced gradually up to 30 and at 35°C no mycelial growth was recorded.

Terefe et al. (2015) studied that the maximum radial growth of *Botrytis fabae* (84.00 mm), the highest conidial size (24.86 × 16.32 µm), sporulation (2.48 × 10^3 conidia/ ml) were recorded at 22 °C while the lowest values of the above parameters were scored at 26 °C. Moreover, the maximum lesion size of 22.83 mm in Bulga-70 and 17.67 mm in 'Degaga' faba bean cultivars was scored on a detached leaf experiment at 22 °C. The highest AUDPC (42.08 mm and 30.92 mm) were calculated in the above cultivars, respectively. Similar results were reported by Czaja et al. (2016), in which the growth and development of *Botrytis* spp. highly affected by temperature. The optimum temperature for mycelial growth and development of the fungi isolated from fig fruit was 18°C. Maximum and minimum temperatures for the fungus development was 38 and 0°C, respectively.

Zehra et al. (2016) stated that the growth and sporulation of *Trichoderma* spp. can be affected by temperature. According to these authors, *T. harzianum* and *T. viride* can tolerate a different range of temperature, they can well grow and sporulated at a temperature range of 20 to 40°C. While the radial growth of and sporulation of *T. hamatum* and *T. asperellum* grew and sporulated better at the temperature ranges of 25-35 °C. Moreover, the mycelial growth was declined at 40 °C and stopped at 45 °C. Morphological variations were observed at different temperatures. The fungus showed a typical compact colony with an irregular margin and scanty sporulation at 40 °C.

As described by Chennappa et al. (2017), *Trichoderma virens*, *Trichoderma viride* and *Trichoderma hamatum* grew faster at the temperature between 25–30 ºC while at 40 ºC, the fungi were growing slowly. Moreover, the maximum dry mycelial weight of *Trichoderma* spp. was obtained at 25 ºC and 30 ºC, the least dry mycelial weight of the fungi was recorded at 40 ºC.

2.2.7. Faba Bean Chocolate Spot Disease Management

Harrison (1984); El-Sayed et al. (2011) stated that regular spraying with benomyl controlled *B. fabae* but had no visible effect on *B. cinerea.* Dithane M45, Galben mancozeb 584 and copper oxychloride were reduced disease severity, incidence, and increased yield and yield components of faba bean. According to Creighton et al. (1985); Mbazia et al. (2016), faba bean chocolate disease can also be controlled by the application of fungicides such as mancozeb, maneb, Benomyl or benzimidazoles. Villegas-Fernández et al. (2009) had also mentioned that the best approach to control chocolate spot is using resistant varieties even though absolute resistance cultivars and or genotypes not available worldwide.

According to El-Hendawy et al. (2010), chemical inducers, KH_2PO_4, $CaCl_2$, ascorbic acid, salicylic acid, and oxalic acid showed varying efficiency in reducing chocolate spot disease severity of faba bean under greenhouse and field conditions, as well as enhanced yield and yield components of the crop. According to El-Sayed et al. (2011) management of faba bean chocolate spot is possible through the application of fungicides, resistant cultivar, biological control agents, and induced resistant and cultural practices. Ramadan and El-Kholy (2014) also mentioned that fungicop 40% EC (copper hydroxide), Oxydor 50% SC (carbendazim), Bitafol Gold3 60 %EC (mancozeb 25% + fosetyl-aluminium 32.5% + cymoxanil 2.5%) and Tridex 80% WP (mancozeb) were found to be effective against chocolate spot caused by *B. cinerea* and *B. fabae*, and the results indicated that *B. fabae* was more sensitive to the above fungicides than *B. cinerea*, both are causing the same disease.

Fernandez-Aparicio et al. (2011) mentioned that intercropping faba bean with cereals like durum wheat (*Triticum durum*), barley (*Hordeum vulgare*), oat (*Avena sativa*) or triticale (*XTriticosecale*) or with legumes: common vetch (*Vicia sativa*) or field pea (*Pisum sativum*) significantly reduced chocolate spot disease. The area under the disease progress curve 52%, 63%, 45%, 44% were reduced by growing faba bean mixed with barley, oats, triticale, and wheat, respectively. As

stated by Terefe (2015), field sanitation, crop rotation, intercropping, reducing crop density, the spatial variation of crop fields, and removing volunteer and alternate hosts are possible management options for this pathogen.

Mbazia et al. (2016) had revealed that the effect of salicylic, citric, ascorbic and oxalic acids as chemical inducers against *B. fabae* in the field and glasshouse conditions and these were significantly decreased the disease severity on the leaves and the stems.

According to GRDC (2017), integrated disease management (IDM) which includes: crop rotation and paddock selection, growing resistant varieties, clean seed and fungicide seed dressings, canopy management through a time of sowing, seeding rate and row spacing, regular crop monitoring, strict hygiene on and off-farm and strategic use of foliar fungicides are a promising method. Moreover, fungicides like carbendazim or procymidone, mancozeb, chlorothalonil as well as copper products may have some efficacy against faba bean chocolate spot.

2.2.7.1. Biological Control of Chocolate Spot Disease

A study conducted by De Melo and Faull (2000) in Spain showed that *Trichoderma* spp. produce several enzymes such as chitinase, cellulase, and beta-1, 3 glucanases that can hydrolyze fungal structures. Hjeljord et al. (2001) reported that *T. harzianum* and *T. viride* as biocontrol agents for a gray mold of grape, apple, and strawberry caused by *B. cinerea*. El-Gindy (2003); Eisa et al. (2006) reported that T. *lignorum, Bacillus subtilis,* and *T. harzianum* reduced the average diameter of *B. fabae* colonies than the control.

According to Elad and Stewart (2004); Fillinger and Elad (2016), in order to germinate and host penetrates, conidia of *Botrytis* spp. need the presence of exogenous nutrients and water. Germination of conidia on the plant surface can be affected by competition for nutrients, inhibitory substances, space, and limitation of saprophytic ability. After infection, the *Botrytis* spp. is affected by host

characteristics that are typical of defending host tissues as occurs with induced resistance.

Mahmoud et al. (2004); Yousef (2008); Hafez (2010) showed that application of *T. harzianum* as foliar treatment effectively reduced disease severity of *B. fabae* or *B. cinerea* on different plant species, including faba bean, lentil and pepper crops. On the other hand, Ibrahim, (2005) found that *T. hamatum* and *T. harzianum* inhibited the growth of two isolates of *Fusarium oxysporum* f.sp. *fabae* isolate.

A study conducted by Eisa et al. (2006) revealed that *T. harzianum, T. virens*, *T. koningii*, *T. viride,* and *B. subtilis* have the ability to inhibit the linear growth of root rot fungi like *F. solani* and *R. solani* on faba bean. Haggag et al. (2006) showed that the antifungal potential of *Trichoderma* against *B. fabae* was evaluated *in vitro* against spore germination and mycelium growth rate and was found effective.

According to Emeran et al. (2006), *Trichoderma harzianum* and *Bacillus subtilis* isolated from healthy faba bean leaf surface were reduced spot diameter and disease severity both under greenhouse and field conditions. A study conducted by Sahile et al. (2009) in Ethiopia also revealed that *Bacillus* isolates found on the surface of faba bean leaves generally showed inhibiting potential in a dual culture against *B. fabae*. The above authors also reported that out of 20 *Bacillus* isolates screened, most of them reduced the development of chocolate spot symptoms caused by *B. fabae* on detached leaves test.

A study conducted by Sahile et al. (2011) revealed that isolates of *T. ovalisporum* and *T. longibrachiatum* as an effective antagonist of *B. fabae* for the first time in Ethiopia. A study conducted in western Algeria by Boubekeur et al. (2012) had also shown *T. viride*, *T. longibrachiatum* and *T. harzianum* tested under laboratory conditions against *B. cinerea* and *B. fabae,* causing faba bean chocolate spot disease had reduced growth and sporulation

A study carried out by Taffa et al. (2013) in Ethiopia confirmed that *Trichoderma* spp. isolates namely: *Trichoderma longibrachiatum, Trichoderma harzianum*, *Trichoderma polysporum*, *Trichoderma koningii*, *Trichoderma citrinoviride* and *Trichoderma spirale, Trichoderma virens* were found to be effective on detached leaf and intact plant tests by reducing diseases severity and incidence.

Ramadan and El-Kholy (2014) conducted an experiment under field conditions. Accordingly, Bio Arc 6.0% WP (*Bacillus megaterium*), BioZeid 2.5% W.P. (*Trichoderma album*) and Plant–Guard (*T. harzianum)* were found to be more effective against *B. cinerea* and *B. fabae*.

As reported by Doni et al. (2014), *Trichoderma* spp. have the potential of enhancing plant growth, protecting the plant against the pathogen. Moreover, they have the capacity to improve root development, and production of auxin and siderophore, and also *Trichoderma* spp. have the ability to increase drought tolerance and salt resistance. A study conducted by Mbazia et al. (2016) showed that *T. viride and T. harzianum* were found to be the best antagonistic agents, even better than the fungicide, Carbendazim, in reducing disease severity caused by *B. fabae* under laboratory (detached leaves), glass-house and field conditions. Fillinger and Elad (2016) reported that biological control can inhibit *Botrytis* spp. by competition for nutrients through reduction of the concentration of nutrients such as carbon, nitrogen and micro-elements results in a reduced rate of germination of the pathogen's conidia and thus the extent of infection.

El-Fiki (2017) suggested that the effect of commercial active dry yeast (ADY) could reduce disease severity and increased yield and yield components like green pods weight per plant, Pods no. /plant, plant height, leaves no. /plant under field conditions. Also, ADY reduced the linear growth of *B. fabae* under in vitro conditions.

Hao et al. (2018) reported that *B. cinerea* has a wide host range and broadest distribution, causing a high economic impact worldwide. The

management of this fungus generally depends on fungicides. Nonetheless, the problem of fungicide resistance enhances the struggle the use of fungicides for *B. cinerea* control. Hence, alternative mycoviruses infecting *Botrytis* spp., such as *Gammaflexiviridae*, *Alphaflexiviridae*, *Narnaviridae*, *Endornaviridae*, *Partitiviridae*, and *Totiviridae* are effective.

3. MATERIALS AND METHODS

The studies were carried out from 2015 to 2018 at Cukurova University, Faculty of Agriculture, Department of Plant Protection, Biotechnology Laboratory as well as under the glass-house and field condition conducted at experimental area of field. They were aimed to determine the morphological, physiological and pathogenic variability of *B. cinerea* isolates; identification of the genetic variation within and among the population of *B. cinerea* and *B. fabae* isolated from Turkey and Ethiopia, respectively. Finally, to evaluate the efficacy of local isolates of *T. harzianum* over a chocolate spot disease of faba bean caused by *B. cinerea* both under *in vitro* and *in vivo* conditions.

3.1. Experimental Materials

3.1.1. Plant Material

Faba bean seed, Rebaya 40, susceptible to chocolate spot (Rhaiem et al., 2002) was used throughout the studies. The cultivar was obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA).

3.1.2. Materials Used for Field Sample Collection

Plastic and paper bags, hand pruner, pruning saw, paper towels, knife, scalpel blades (10, 11, 20 and 22 number), scalpel blade handle, pencil labels, camera, alcohol (96% and 70%) disinfectant for hands and tools were used during sample collection from the fields.

3.1.3. Materials Used for Classical Studies

After the samples were brought to the laboratory, isolation of *Botrytis* spp. and *T. harzianum* were carried out using sterile Petri plate (9 cm in diameter), alcohol, sodium hypochlorite (NaOCl), potato dextrose agar (40 g/L; D (+) Glucose 20 g/L; Agar-agar 15 g/L) (Merck 1.10130). Moreover, antibiotics like

tetracycline (100 mg/L) (Sigma: T3258-5G (PDA-tet) and streptomycin (100 mg/L (Duchefa, (PDA-tet) were used where necessary; and all cultural media were autoclaved (Hirayama) before use as described by Endes (2017).

Potato dextrose agar (PDA) was used for a morphological study of *B. cinerea*. However, for inducing sporulation of the fungus, faba bean leaf dextrose agar (250 g/L of faba bean leaves + 30 g of dextrose + 20 mg of agar + 20 g of sodium chloride) in 1 L of distilled water was used (El-Hendawy et al., 2010). Faba bean leaves were also used for conidiophore characterization. For long-term storage of both fungi, 2 ml Eppendorf tube, sterile water and 30% of sterilized glycerol (Merck 104092) as well as -20 and -80 °C deep freezers were used. In order to prepare fungal specimens for microscopic examination, sterile water, 30 % of glycerol, Lactophenol (Merck 113741) was used where necessary. In addition to this, light microscopes (Leica, Model DME and Olympus, BX51) at different magnification, 10x, 20x, 40x and 100x and digital camera were used. For the evaluation of the different temperature effect on mycelial growth of *Botrytis* and *T. harzianum*, incubator (Nüve, ES 500) was used (Endes, 2017).

3.1.4. Materials Used for the Molecular Studies

For DNA extraction from *Botrytis* and *Trichoderma* species: tissue grinder, water bath (Nüve, NB9), two centrifuges (Sigma, 2K15 and Nüve, NF048), automatic pipets with different volumes (10, 20, 100, 200 and 1000 μm) were used. Moreover, three primers with different gene regions, internal transcribed spacer (ITS5/ITS4) (White et al., 1990), β-tubulin (Bt2a/Bt2b) (Glass and Donaldson, 1995) and translation elongation factor one-alpha (tef71 and tef997) (Shoukouhi and Bissett, 2009) as well as ProFlex ™ PCR System (Applied Biosystems, Milan, Italy) were used for polymerase chain reaction study. For electrophoresis study, a different model of tanks (Wide Mini-Sub Cell GT Cell, Sub-Cell® GT Cell and Biorad, mini-Sub® GT Cell), 1XTAE buffer solution, Agarose gel (Sigma, A6013),

ethidium bromide (Sigma, E1510) and UVITEC imaging system (Cambridge, UK) were used. PCR purification kit (Qiagen, 28106) was used. For glasshouse and field studies, plastic pots (14 cm in diameter), white polyethylene sheet (5 m ×15 m), mixtures of soil (peat/clay/sand 2:2:1 (v: v: v)) were used.

3.2. Methods

3.2.1. Isolation of *Botrytis* Species

Flowers, leaves and stems showing chocolate spot, dark brown, and gray mold symptoms were collected from faba bean (*Vicia faba*) starting from flowering to pods harvesting stages in Adana province, Turkey, from late December to early March 2015/16 and 2016/17, during main growing seasons. In each faba bean field, six to seven diseased aerial plant parts were collected randomly, then the samples were placed in a plastic bag and labeled with information of collection sites and origin used in further analysis as described in Figure 3.1 and Table 3.1. In addition, *B. fabae* isolates were obtained from the Post Graduate Laboratory, Plant Protection Department, Haramaya University, Ethiopia, which were isolated in 2014.

After the samples brought to Department of Plant Protection, Biotechnology Laboratory, they were carefully washed with tap water; the roots were removed and sterilized with 70% alcohol for 10 seconds and subsequently washed in 1% sodium hypochlorite for 2-3 minutes. Sterilized pieces were rinsed with sterile distilled water several times so as to remove the remaining disinfectant solution and dried on the sterile Whatman filter papers (Aldesuquy et al., 2014).

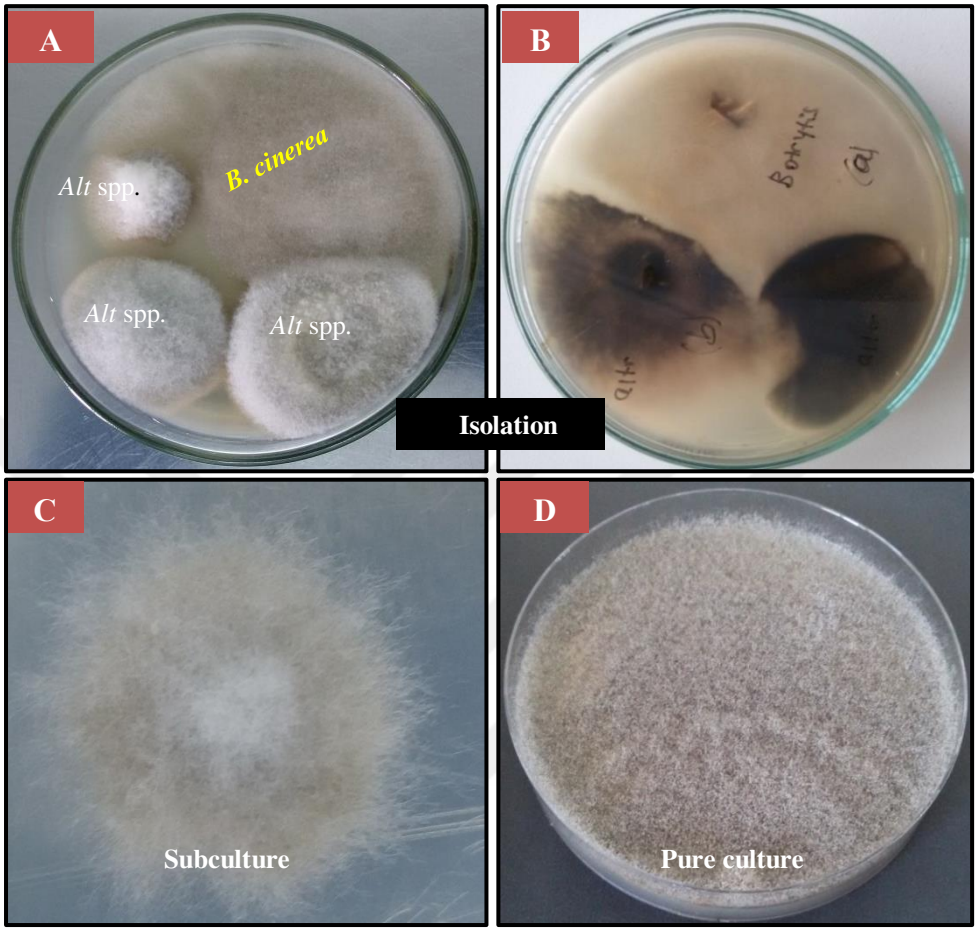

Figure 3.1. Isolation (A, B) and subculture of *B. cinerea* (C, D) on Potato Dextrose Agar medium, causing chocolate spot disease on faba bean, during 2015/16 and 2016/17 main cropping seasons

The dried samples were cut off into approximately 3-4 mm^2 between the infected and the healthy site by using the sterile scalpel to prevent any other contamination. The tissues were transferred onto potato dextrose agar medium in a Petri dish (Merck, Germany) supplemented with Tetracycline antibiotics (100 mg/L) and incubated for 3 to 4 days under a room temperature of 20±2 °C. Mycelium from the growing edge was subcultured to new PDA plates until getting

pure culture (Figure 3.1). Overall activities were conducted in a sterile laminar flow.

Table 3.1. List of isolates of *Botrytis* spp., causing faba bean chocolate spot disease, collected in the Adana province of Turkey and Haramaya, Ethiopia during 2015/16 and 2016/17 cropping seasons

Isolates code	Collected Season	Pathogen isolates	Diseased organ
Bc1	2015/16	*B. cinerea*	Leaf
Bc2	2015/16	*B. cinerea*	Leaf
Bc3	2015/16	*B. cinerea*	Leaf
Bc4	2015/16	*B. cinerea*	Leaf
Bc5	2015/16	*B. cinerea*	Leaf
Bc6	2015/16	*B. cinerea*	Leaf
Bc7	2015/16	*B. cinerea*	Stem
Bc8	2015/16	*B. cinerea*	Stem
Bc9	2016/17	*B. cinerea*	Leaf
Bc10	2016/17	*B. cinerea*	Leaf
Bc11	2016/17	*B. cinerea*	Leaf
Bc12	2016/17	*B. cinerea*	Leaf
Bc13	2016/17	*B. cinerea*	Leaf
Bc14	2016/17	*B. cinerea*	Leaf
Bc15	2016/17	*B. cinerea*	Leaf
Bc16	2016/17	*B. cinerea*	Leaf
Bc17	2016/17	*B. cinerea*	Leaf
Bc18	2016/17	*B. cinerea*	Leaf
***Botrytis fabae* (used only in the molecular study)**			
Bf1	2014/15	*B. fabae*	Leaf
Bf2	2014/15	*B. fabae*	Leaf
Bf3	2014/15	*B. fabae*	Leaf
Bf4	2014/15	*B. fabae*	Leaf
Bf5	2014/15	*B. fabae*	Leaf
Bf6	2014/15	*B. fabae*	Leaf
Bf7	2014/15	*B. fabae*	Leaf
Bf8	2014/15	*B. fabae*	Leaf

Bc=*Botrytis cinerea*; Bf=*Botrytis fabae*

3.2.2. Isolation *Trichoderma harzianum*

Isolation of *T. harzianum* from faba bean and field pea was carried out as follows: leaves and stems infected by either biotic or abiotic diseases were selected.

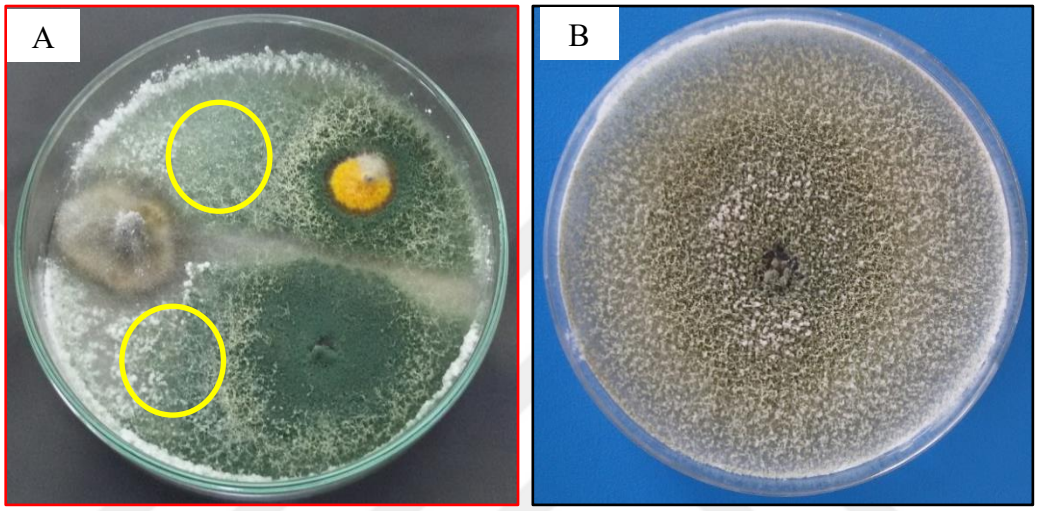

Figure 3.2. Isolation (A) and subculture (B) of *Trichoderma harzianum* on Potato dextrose agar media during 2015/16 and 2016/17 main cropping seasons

Then, rinsed three to four times with sterile distilled water. Sterilized pieces were blotted dry on the sterile Whatman filter paper. The dried samples were cut off into about 5 mm^2 in the infected site by using the sterile scalpel. The tissues were put onto tetracycline amended Potato Dextrose Agar medium in a Petri dish (Merck, Germany) and incubated for 2 days at 25 °C. Then, fungal mycelia emerging first and growing quickly were transferred to a new PDA medium and purification was continued until getting pure culture (Figure 3.2 and Table 3.2). Colony growth pattern of *Trichoderma harzianum* under light and dark conditions was shown in figure 3.3. All the isolates were identified based on morphological structures.

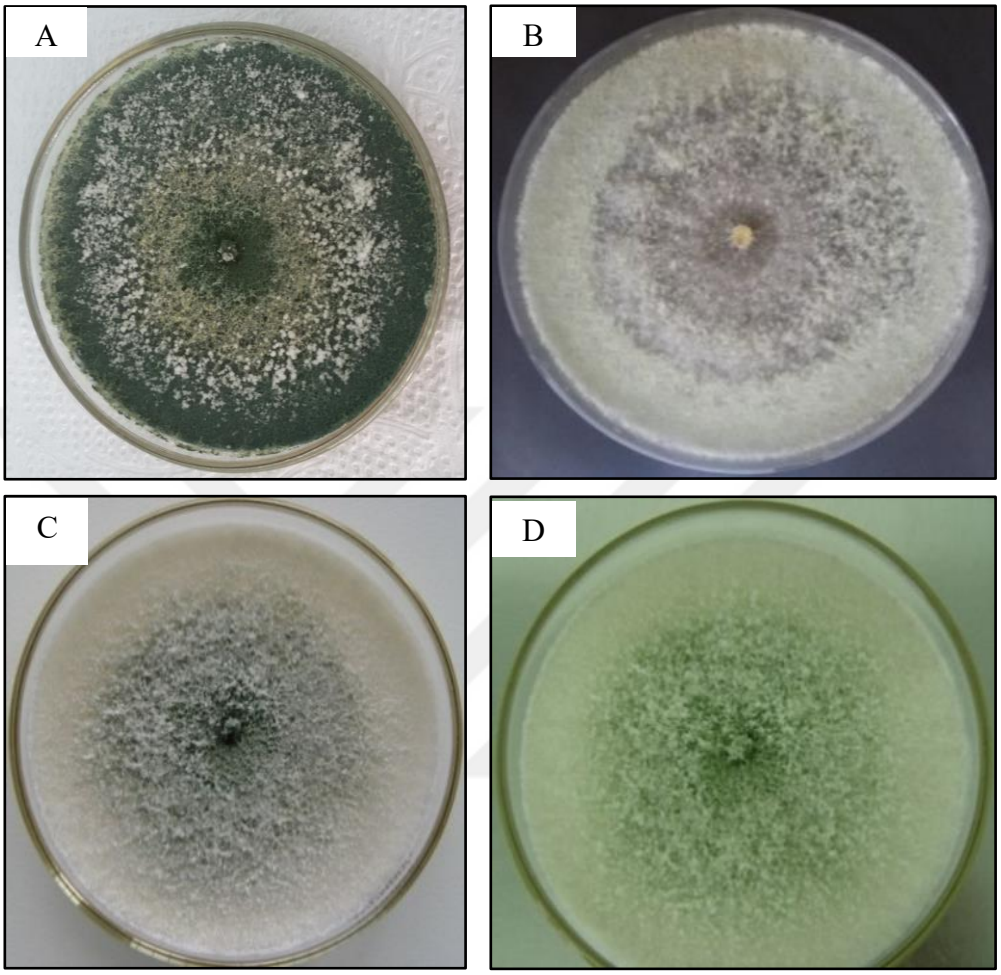

Figure 3.3. Colony appearance of *Trichoderma harzianum* grown on Potato dextrose agar media under the light (A and B) and dark conditions (C and D)

Table 3.2. List of antagonistic *Trichoderma harzianum* collected from Adana and Mersin provinces

Isolates code	Bio-agent isolate	Substrates	Crop parts
T1	*T. harzianum*	Faba bean	Leaf
T2	*T. harzianum*	Faba bean	Leaf
T3	*T. harzianum*	Faba bean	Leaf
T4	*T. harzianum*	Faba bean	Stem
T5	*T. harzianum*	Field pea	Leaf
T6	*T. harzianum*	Field pea	Leaf
T7	*T. harzianum*	Faba bean	Leaf
T8	*T. harzianum*	Faba bean	Leaf
T9	*T. harzianum*	Faba bean	Leaf
T10	*T. harzianum*	Faba bean	Stem
T11	*T. harzianum*	Faba bean	Leaf
T12	*T. harzianum*	Faba bean	Leaf
T13	*T. harzianum*	Faba bean	Leaf
T14	*T. harzianum*	Faba bean	Leaf
T15	*T. harzianum*	Faba bean	Leaf
T16	*T. harzianum*	Faba bean	Leaf
T17	*T. harzianum*	Faba bean	Leaf
T18	*T. harzianum*	Faba bean	Leaf
T19	*T. harzianum*	Faba bean	Stem
T20	*T. harzianum*	Faba bean	Stem

3.2.3. Single-Spore Isolation and Stock Culture Preparation

The single spore technique was adopted to obtain a pure culture. Accordingly, 4 mm *B. cinerea* and *T. harzianum* cultures were cut with a sterile scalpel and placed on a Petri plate contain potato dextrose agar and kept under a dark condition at room temperature for 12 days. Using a sterilized loop, very few amounts of conidia were suspended in 1500 µL Eppendorf containing sterilized water for 1 to 3 min and shacked gently on vortex several times. A hundred microliters of the spore suspensions were spread onto PDA medium 9 cm plate and incubated for 24-48 hours at 20°C under 12 h dark and 12 light conditions.

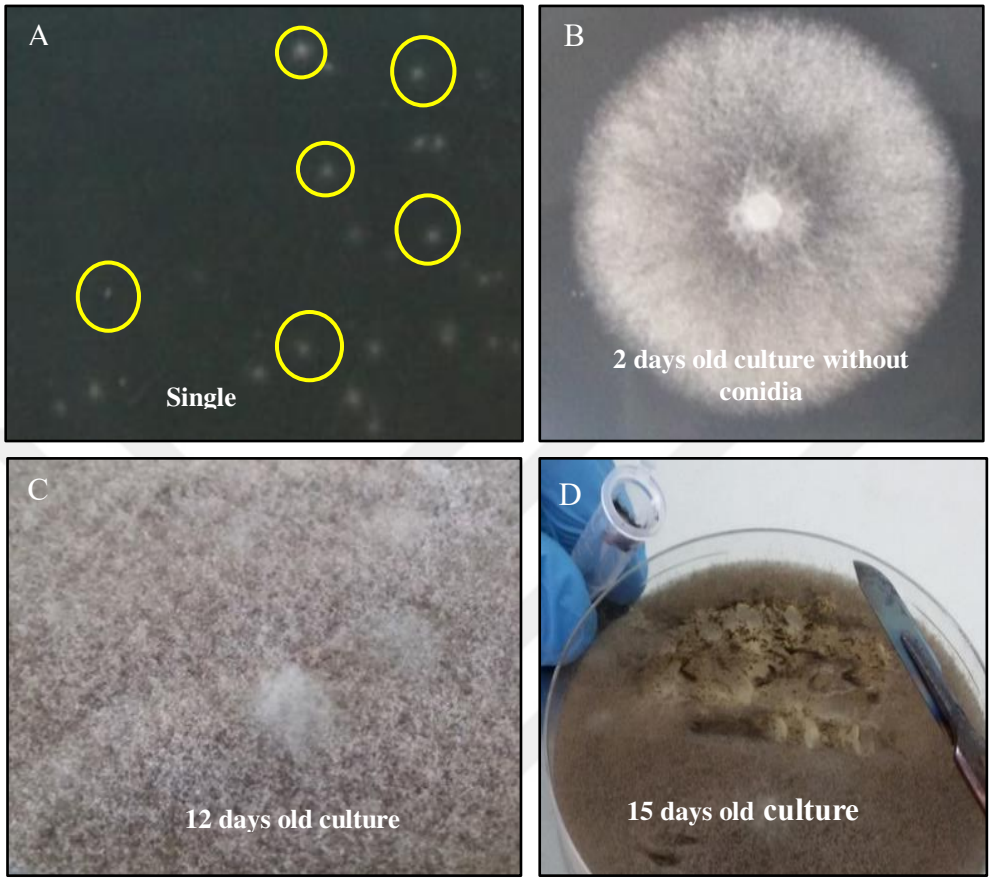

Figure 3.4. Single-spore study and stock culture preparation for *Botrytis* spp.

Each mycelial colony from a single spore was transferred to a new PDA and cultured at 20 to 23 °C (Figure 3.4). A piece of agar containing the mycelia from the eight to ten days culture was cut from the media were kept in 2 ml Eppendorf tubes containing 30% of sterilized glycerol and they were stored at -20 and -80 deep–freezers for future use (Figure 3.5).

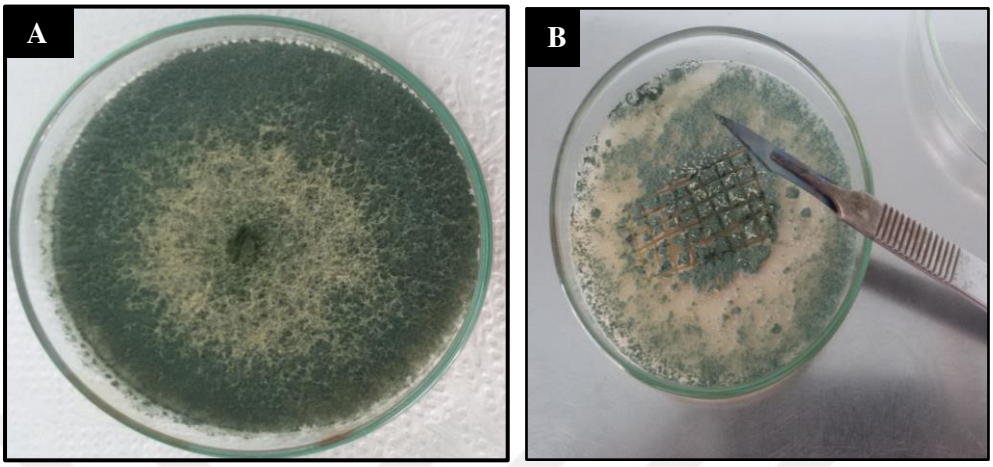

Figure 3.5. Stock culture preparation for isolates of *Trichoderma harzianum*; pure *T. harzianum* culture (A), cutting of mycelia with a sterile scalpel (B)

3.2.4. Colony Growth and Morphological Characterization of *B. cinerea*

Colony growth and conidial as well as conidiophore characteristics of the *B. cinerea* isolates were determined on potato dextrose agar (PDA) medium after 15 days incubation under 12 h dark and 12 h light condition at 20±2 °C. For these studies, 4 mm in diameter of 3 days old actively growing mycelium cultures were transferred to new PDA. The fungus colony structures like colony texture, shape and color were observed and recorded 2, 6 and 15 days after inoculation as described by Hosen et al. (2010). In addition, morphological features of conidia and conidiophores of the fungus were determined on the same medium and temperature after 21 days. Consequently, the length and width of 50 conidia were measured at 100 × magnification lens (Leica, Model DME) as well as the ratio of length to width, mean, size and shape were determined. The experiment was repeated twice.

To induce sclerotia formation, the dishes were separately incubated at room temperature of 17±2 °C in the dark for 20 days. A total of 30 sclerotia produced by each isolate was chosen randomly, and length and width were

measured using a scientific ruler. Moreover, the ratio of length to width, mean, size, number per Petri dish, shape and color of sclerotia were determined.

3.2.5. Molecular Identifications of *Botrytis* and *Trichoderma* fungi

3.2.5.1. Extraction of DNA from Botrytis spp.

A pure culture of eight of each *B. cinerea* (Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7, Bc8) and *B. fabae* (Bf1, Bf2, Bf3, Bf4, Bf5, Bf6, Bf7, Bf8) were used throughout this study. DNA extraction was performed following the Cenis (1992) protocol with slight modification. Mycelia obtained from single conidia were cultured in PD medium at 20±2 °C for 7 days under dark conditions. Approximately 100 mg of fungal mycelia was scraped using sterile surgical scalpel blade number 10 (Wuxi Xinda Medical Device, China) and transferred to 1.5 ml centrifuge tubes, incubated at -20 °C for a night. The mycelia were grounded with a sterile tissue grinder until becoming a fine powder, 500 µL extraction buffers (200 mM Tris-HCI; 250 mM NaCl; 25 mM EDTA; % 2 SDS (Sodium dodecyl sulfate; pH 8.5)) was added to centrifuge tubes. Then, 150 µL of 3 M sodium acetate (pH 5.2) was added, and incubated at -20 °C for ten minutes. The tubes were centrifuged at 14.000 rpm for 10 minutes and the supernatant was transferred to another sterile centrifuge tube. An equal volume of isopropanol was added and incubated at -4 °C for 10 minutes, then precipitated DNA was pelleted by centrifugation at 14.000 rpm for 10 minutes. Finally, they were washed with 1000 µL of 70% ethanol, air dried at room temperature for 40 minutes, and re-suspended in 40 µL of TE buffer, and incubated at -20 for future use.

3.2.5.2. Extraction of DNA from Trichoderma harzianum

Extraction of genomic DNA from *T. harzianum* was carried out using the Cetyltrimethylammonium bromide (CTAB) based on the protocol proposed by Stirling, (2003); Sadfi-Zouaoui et al. (2009); Umesha et al. (2016). The fungal spores were grown in liquid PDA medium at 28 °C for 48 h under continuous

shaking on a rotary shaker (125 rev/min). Mycelia were harvested on cheesecloth in a glass beaker, washed 3 to 4 times with sterile water to remove a growth medium and colored compounds, which may inhibit the quality of DNA, and then transferred into a mortar, liquid nitrogen was added, frozen, and lyophilized, and they were then pulverized with a pestle. Five hundred (500 µL) CTAB extraction buffer was added to the 1.5 ml sterile centrifuge tubes that contain 100 mg of the powdered mycelial, the tubes were vortexed to mix thoroughly, making sure that all the mycelium was suspended in the lysis buffer. They were then incubated at 65 °C for 40 minutes in a water bath, gently mixed every 2 mins. An equal volume (500 µL) of chloroform was added to each tube, the mixture was homogenized, and spun in a centrifuge at 14,000 rpm for 10 minutes (Figure 3.6). The aqueous phase (top layer) was transferred to another sterile centrifuge tube. An equal volume of isopropanol was added, and they were mixed and incubated at 4 °C for 10 min for precipitation of the DNA. Then they were centrifuged at 14,000 rpm for 10 minutes and the supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol twice, air dried at room temperature, and the purified DNA was suspended in TE buffer (30 µL) and kept at -20 °C until use.

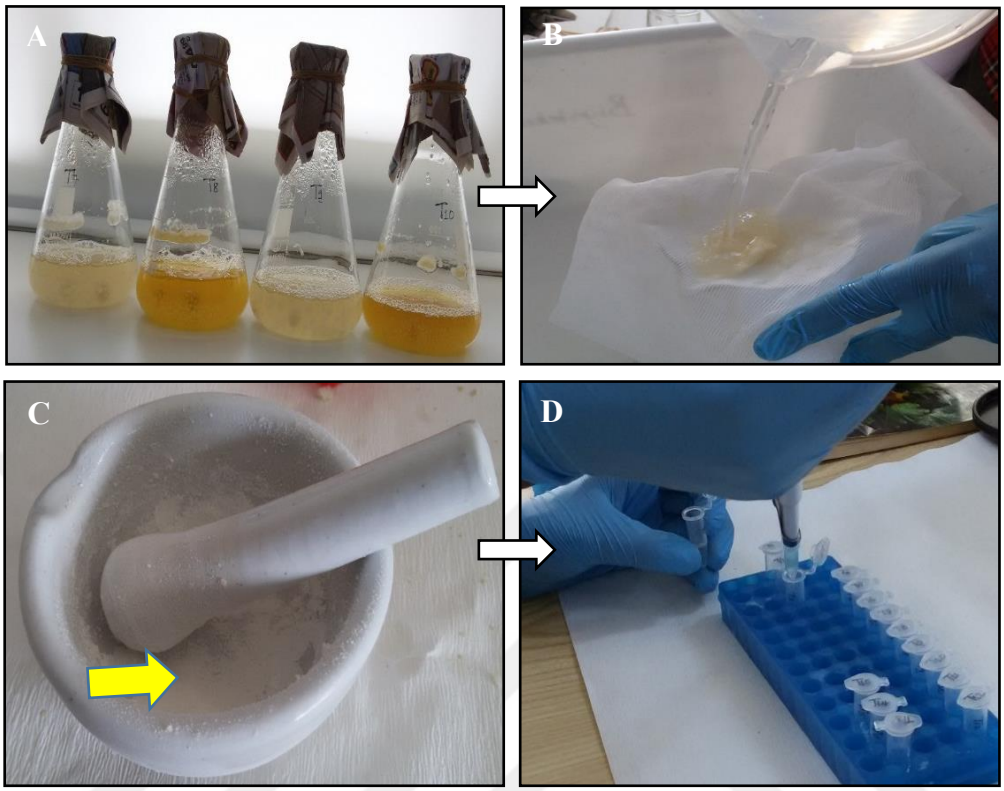

Figure 3.6. Extraction of DNA from *Trichoderma* using CTAB method; the culture of *T. harzianum* spores in liquid PDA (A), washing mycelia with sterile water so as to remove coloring compounds (B), grinding of lyophilized mycelia with mortar and pestle (C), adding extraction buffer to the Eppendorf (D)

3.2.5.3. PCR Reactions

For identification *Botrytis* species (*B. cinerea* and *B. fabae*), the universal primers, the internal transcribed spacer (ITS5 and ITS4) and β-tubulin (Bt2a and Bt2b) were used. The master mix comprised of: 25 mM MgCI2, 10× buffer, 10 mM dNTPs, forward primers, reverse primers, H_2O, Taq Polymerase and template DNA. The primer sequences and their volumes were described in Table 3.3, 3.4 and 3.5. For internal transcribed spacer gene region, thermocycler was programmed with an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 45 second, primer annealing at 55 °C for 45 second

and 72 °C for 1.50 minutes and an extension at 72 °C for 6 minutes. However, for that beta-tubulin gene region, it was programmed with an initial denaturation at 95 °C for 75 second, followed by 40 cycles of denaturation at 95 °C for 45 second, primer annealing at 58 °C for 45 second and 72 °C for 1.50 minutes and primer extension at 72 °C for 6 minutes.

Table 3.3. Lists of primers used for PCR amplification and sequencing

Primer name	Primer sequence (5'- 3') orientation	Direction	References
ITS5	GGAAGTAAAAGTCGTAACAAGG	Forward	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	Reverse	
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Forward	Glass and Donaldson (1995)
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	Reverse	
tef71	CAAAATGGGTAAGGAGGASAAGAC	Forward	Shoukouhi and Bissett (2009)
tef997	CAGTACCGGCRGCRATRATSAG	Reverse	
tef85	AGGACAAGACTCACATCAACG	Forward	
tef954	AGTACCAGTGATCATGTTCTTG	Reverse	

Trichoderma isolates were identified using the universal primer, ITS and single-copy protein-coding genes, translation elongation factor (*tef1*- α). The above master mix protocol was used. The primer sequences and their volumes were described in Table 3.3. and 3.5. For translation elongation factor (tef71 and tef997), DNA amplification protocol was as follow: initial denaturation for 6 min at 95 °C, then followed by 4 cycles at 95 °C each of 60 sec, 1.30 min at 70°C, and 1.30 min at 72°C, followed by twenty six cycles with the attachment (annealing) temperature decreasing from 68 °C to 55 °C (a gradient of 0.5 °C reduction per cycle), followed by 12 cycles with the annealing temperature at 55 °C and a final extension of 7 min at 72 °C, primer pairs, tef85 and tef954, were employed for sequencing of *Trichoderma* nucleotides (Braithwaite et al., 2017; Shoukouhi and Bissett, 2009). The PCR was carried out on a ProFlex PCR System_(Applied Biosystems, Milan, Italy).

Table 3.4. Reagent and master mix for *Botrytis* species

Reaction mixture		Volume (µL)	
Internal transcribed spacer (ITS5/ITS4)	β-tubulin (Bt2a/Bt2b)	1× (25)	×4 (100)
10× Buffer	"	2.5	10
25 mM MgCl2	"	2.5	10
10 mM dNTP	"	0.5	2
ITS5 (f)	Bt2a (f)	0.5	2
ITS4 (r)	Bt2b (r)	0.5	2
DNA	"	2	8
Taq Polymerase	"	0.2	1
H2O	"	16.3	65.3

Table 3.5. Reagent and Master Mix for *Trichoderma* species

TEF1			ITS		
Reaction mixture	Volume (µL)		Reaction mixture	Volume (µL)	
	×1 (25)	×24 (600)		×1 (25)	×24 (600)
25 mM $MgCl_2$	2.5	60	25 mM $MgCl_2$	2.5	60
10× PCR Buffer	2.5	60	10× PCR Buffer	2.5	60
tef71f	0.5	12	ITS5f	0.5	12
tef997r	0.5	12	ITS4r	0.5	12
dNTP 10 mM	0.5	12	dNTP 10 mM	0.5	12
Template DNA	1	24	Template DNA	2	48
Taq Polymerase	0.2	4.8	Taq Polymerase	0.2	4.8
H_2O	17.3	415.2	H_2O	16.3	391.2

3.2.5.4. Agarose Gel Electrophoresis

The amplified *Botrytis* and *Trichoderma* species' nucleic acids (containing 3 µL DNA + 2 µL dyes + 5 µL H_2O) were separated on 1 % agarose gel electrophoresis in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA at pH 8). A molecular-weight size marker (12 µL), DNA ladder, with 200 bp (Thermo Fisher Scientific) was used to identify the approximate sizes of amplified products run on a gel during electrophoresis. It was run at 80 voltage for 1 h and 20 minutes, stained with ethidium bromide for 35 minutes, then revealed and photographed over a UVITEC imaging system (Cambridge, UK).

3.2.5.5. DNA Sequencing and Phylogenetic Analysis

DNA purification was conducted using QIAquick (QIAGEN) PCR purification kit as follows:

1. Five volumes of buffer PB was added to 1 volume of the PCR sample and mix.
2. Ten microliters of 3 M sodium acetate was added to PB as a pH indicator, then the color of the mixture became yellow.
3. A QIAquick spin column was placed in a provided 2 ml collection tube.
4. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 minute.
5. Flow-through was discarded. The QIAquick column was placed back into the same tube.
6. To wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuge for 1 minute.
7. Flow-through discarded and the QIAquick column was placed back in the same tube. Centrifuge the column for an additional 1 min.
8. QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1 min.

For this study, 46 and 44 µL of each sample of *Botrytis* species and *Trichoderma* species, respectively, were sent to the GENOKS Company (Genetik Hastalıklar Tanı Merkezi/Genetic Diseases Diagnostic Center, Ankara) for sequencing. The universal primer of the internal transcribed spacer (ITS) regions and partial gene sequences of translation elongation factor1-α (tef1-α) were sequenced for *T. harzianum* isolates. Moreover, ITS and Beta-tubulin gene regions were sequenced for *Botrytis* species.

Sequences were edited with the BioEdit sequence alignment editor software version 7.2 (Saito et al., 2016). ClustalW tool was used for homologous sequences alignment (for pairwise alignment: gap opening penalty 15 and gap extension penalty 6.66; for multiple alignments: gap opening penalty 15 and gap extension penalty 6.66). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993) and the 1000 repetition bootstrap was employed.

3.2.6. Effect of different Temperature on Mycelial Growth of *B. cinerea* and *T. harzianum*

Colony diameter and radial growth rate (RGR) of eight isolates of *Botrytis cinerea* (Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7 and Bc8) and twelve *T. harzianum* isolates (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11 and T12) were determined on the PDA as stated by Carrión et al. (2016). Agar plugs (4 mm diameter) containing mycelia of 4 days old *B. cinerea* and 2 days old *T. harzianum* were by using sterile cork borer and inoculated to the center of 90 mm diameter Petri plate, one plug per dish (Figure 3.7). The experiments were subjected to a complete randomized design (CRD) with three replications. After putting inside sterile plastic boxes, the whole dishes were incubated separately at 5, 10, 15, 20, 25, 30 and 35 °C in the dark for *B. cinerea*, and 5, 10, 15, 20, 25, 30, 35 and 40 °C for *T. harzianum*. The colony diameter of each dish was measured in two perpendicular directions every 24 h until the entire Petri plate was covered by the mycelia. Mycelial growth was measured daily using a scientific ruler. The radial growth rate was calculated using the formula RGR (mm/day) = (D3-D2) /2. Where, D2 and D3 are the colony diameters of 2 and 3 days old in each Petri plate, respectively (Zhong et al., 2016).

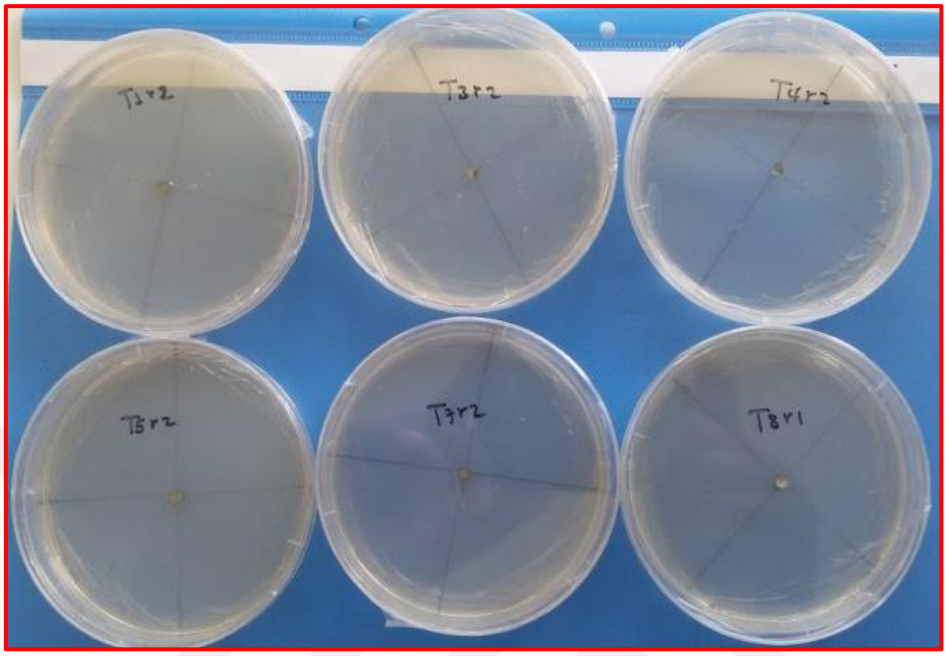

Figure 3.7. Effect of temperature on mycelial growth of *B. cinerea* and *T. harzianum* isolates

3.2.7. Pathogenicity Assay

Variability among the *B. cinerea* isolates was determined both on detached leaves and intact plant, under laboratory and glass-house conditions, respectively.

3.2.7.1. Pathogenicity Test on Detached Faba Bean Leaves

Faba bean seeds (Rebaya 40) were grown in pots at Plant Protection Research Station, Cukurova University, on October 20, 2016. The leaves were collected from 65 days old seedlings as described by Zhang et al. (2010) with minor modification. Then, leaflets were disinfected with 1% of sodium hypochlorite for 2 min and washed in sterile distilled water several times. Leaflets were placed face up on moistened paper towels on a Petri plate. Three days old agar plugs containing *B. cinerea* isolates (Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7, Bc8), 4 mm diameter, were removed by sterile cork borer and placed on the leaflets. Three leaflets from the different faba bean plant were used for each

isolate. Faba bean leaflets inoculated with pathogen free disk plugs PDA were served as controls. The experiment was set in a complete randomized design with three replicates. The plastic boxes were incubated at 20±2°C in the dark for 12 days. Re-isolation was performed for all isolates and their cultural and morphological characteristics were similar to the one isolated from the field, then Koch's postulates were confirmed. The length and width diameters of the lesions formed around each agar plug on leaflets were measured starting from 5 days after inoculation until mycelia completely covered the leaflets.

3.2.7.2. Pathogenicity Test on Intact Faba Bean Plant

A separate experiment was carried out on an intact faba bean to determine virulence variability among ten *B. cinerea* isolates. Disease severity and area under the disease progress curve were assessed. Under this study, isolates that had shown the promising result in the above experiment, as well as other, were tested so as to confirm the results obtained in the detached leaf. Conidia were used as an inoculum source for whole faba bean plants. The faba bean seeds were planted in plastic pots (14 cm in diameter) containing peat/clay/sand 2:2:1 (v: v: v) soil mixture at the glass-house of Plant Protection Research Station, Cukurova University. In each pot, five seeds per pot were sown and thinned to 3 seedlings after 15 days. Forty (40) days old faba bean seedlings were inoculated with the most virulent *B. cinerea* (Bc1, Bc7, Bc8, Bc10, Bc11, Bc13, Bc14 and Bc17) isolates with spore suspensions of 2.5 x 10^5 conidia/ml. Another faba bean seedlings were sprayed with sterilized water which served as a control, and then the pots were covered with white polyethylene sheets after supported by metal frames (Tivoli et al., 2006). As night and day temperature ranges were high, the sheets were not removed for 5 days. The experiment was arranged in a completely randomized design (CRD) and replicated three times. Chocolate spot symptom was scored three times using a 1-9 scale according to ICARDA (1986) (Table 3.6) starting from disease onset (one week after inoculation) and disease incidence was recorded 21 days after inoculation.

Table 3.6. Faba bean chocolate spot severity score scales under *in vivo* conditions

(1–9) Rating Scale	Disease Percentage
1	No lesions or a few small browns covering up to 1% of the leaf surface
3	Few small discrete brown circular non-sporulating lesions (2–3 mm in diameter) covering 1–2% of the leaf surface
5	Lesions common (3–5 mm in diameter) some coalesced covering 2–5% of leaf surface with some defoliation and very poor sporulation
7	Large coalesced irregular blackish sporulating lesions that cover 5–10% of leaf surface average defoliation and flower drop and some dead plants
9	Extensive large coalesced heavily sporulating lesions covering more than 10% of the leaf surface with severe defoliation stem girdling and death of the great majority of plants

Prior to analysis, disease severity was converted to a severity index (SI) as described by Wheeler (1969):

$$\text{Severity index (\%)} = \frac{\sum (\text{no. of the plant in category} \times \text{category value}) \times 100}{\text{Total no. of plants} \times \text{max. category value}}$$

To assess the progress of the symptoms over time, the area under the disease progress curve (AUDPC) was calculated using the following formula (Steffenson and Webster, 1992):

$$\text{AUDPC} = \sum_{i=1}^{n} (0.5(xi + 1 + xi)\,(ti + 1 - ti))$$

Where n is a total number of assessment times, ti is a time of the i^{th} assessment in days from the first assessment date, xi is a percentage of disease severity at i^{th} assessment.

3.2.8. Biocontrol Efficacy of *T. harzianum* against *B. cinerea*

A study of the biological control was conducted to test indigenous *T. harzianum* isolates ability to control *B. cinerea,* the causal agent of faba bean chocolate spot under *in vitro* and *in vivo* conditions.

3.2.8.1. Dual Culturing Method

Two methods were employed for dual culture techniques (Figure 3.8). Evaluation of the antagonistic ability of *T. harzianum* on *B. cinerea* isolates was followed by Sahile (2008) and Rahman et al. (2009). In the 1st method, 5 days old agar plugs from the edges of an actively growing containing *B. cinerea* was cut with a sterilized cork borer (4 mm) and placed at the edge of the potato dextrose agar (PDA) medium plate (90 mm in diameter). At the opposite end of the same Petri plate, agar disk of equal size of *T. harzianum* was placed. In the 2nd method, *Trichoderma* (T) was placed 2 cm away from the edge of the Petri dish, and *B. cinerea* (B) was similarly placed 2 cm away from the edge of the Petri dish on the opposite side. On a control plate, *B. cinerea* alone was placed in the same manner on a fresh PDA plate. All pairings were incubated at 23 °C in the dark for two weeks. Antagonistic ability was tested by measuring the radius of the *B. cinerea* isolates colony in the direction of the antagonist colony (T) and the radius of the *B. cinerea* isolates colony in the control plate (B). In addition, inhibition zones and the number of days of overgrowth *B. cinerea* colony were measured. The two readings were transformed into percentage inhibition of radial growth (PIRG) using the following formula (Skidmore and Dickinson, 1976). Treatments were arranged in CRD factorial, three replications were employed for both methods. The experiment was repeated twice.

$$\text{Inhibition of Radial Growth (\%)} = \frac{C-D}{C} \text{x}100$$

Where C: the linear growth of *B. cinerea* grown alone; D: linear growth of *B. cinerea* in presence of *T. harzianum*

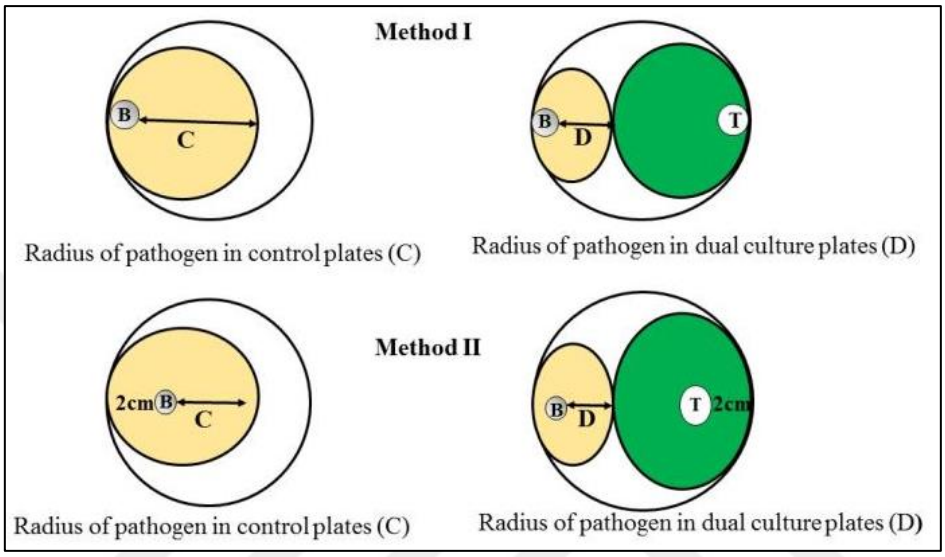

Figure 3.8. Model for dual culture test of *T. harzianum* and *B. cinerea* (Rahman et al., 2009)

3.2.8.2. Antagonistic Evaluation Using Detached Leaves

Methods developed by Ellwood et al. (2006) were followed with slight modification. Ten isolates of *T. harzianum* showed promising antagonistic potential in a dual culture (T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10) were used. The faba bean seeds, Rebya 40 were grown at the field site of Cukurova University (C.U), Plant Protection Research Site. Leaves similar in physiological ages were detached from 6 to 7 weeks old faba bean plants, from the middle nodes. After bringing to the laboratory, they were surface disinfected in 1% sodium hypochlorite for 2 min, subsequently rinsed with distilled sterile water three times and allowed to dry on sterile Whatman filter paper. The sterile filter papers were put in the Petri dishes and moistened with distilled sterile water. The spore suspension of *B. cinerea* and antagonistic *T. harzianum* isolates were prepared by

pouring 10 ml of sterile water onto each dish where the pathogen was growing, and dislodging the spores by scraping the surface of the medium with a sterile scalpel. This suspension was filtered through two layers of sterile cheesecloth, adjusted to 2.5 x 10^5 spores /ml (El-Banoby et al., 2013). Then, one drop (10 µL) of spore suspension was placed near the middle of the leaves and incubated at room temperature for 4-5 hrs. Then, an equal volume (10 µL) and similar spore concentration (2.5 x 10^5 spores /ml) of the pathogenic fungus was added to the same place, where the drop of the antagonistic fungal spore suspension was placed. Detached leaves (two leaves per plate) inoculated only with *B. cinerea* isolates were used as a control. The experiment was arranged in a CRD with three replications and repeated twice. The Petri plates were kept inside the plastic box after spraying with sterile water (to induce relative humidity) and incubated at 21 °C as described by Taffa et al. (2013) with minor modification. Disease severity was scored on the leaves using a 1-4 scale as described in Table 3.7. In addition, the area under the disease progress curve (AUDPC) was calculated from the three percentage severity index.

Table 3.7. The disease severity was rated using the following a 1–4 scale (Tivoli et al., 2006) under *in vitro* conditions

1–4 scale	**Descriptions**
1	No infection or very small flecks (1–25% necrosis)
2	Necrotic flecks with few small lesions (26–50% necrosis); and very poor sporulation
3	Medium coalesced lesions (51–75% necrosis) with intermediate sporulation
4	Large coalesced lesions (76–100% necrosis) with abundant sporulation

3.2.8.3. Antagonistic Assessment under Glass-House Conditions

Susceptible faba bean seeds, which were taken from the ICARDA sown in plastic pots filled with sand, manure, and compost in 1:2:2 ratios. Randomized complete block design (RCBD) factorial was adopted for this experiment, the

effect of *T. harzianum* isolates on disease. Disease severity, incidence and area under disease progress curve values were analyzed in two ways. Treatment was repeated twice and replicated thrice. The spore suspension of *T. harzianum* isolates was prepared, six weeks old faba bean plants were sprayed with 20 ml/plant at a concentration of 2.5×10^5 spores/ml (Mohammed et al., 1994).

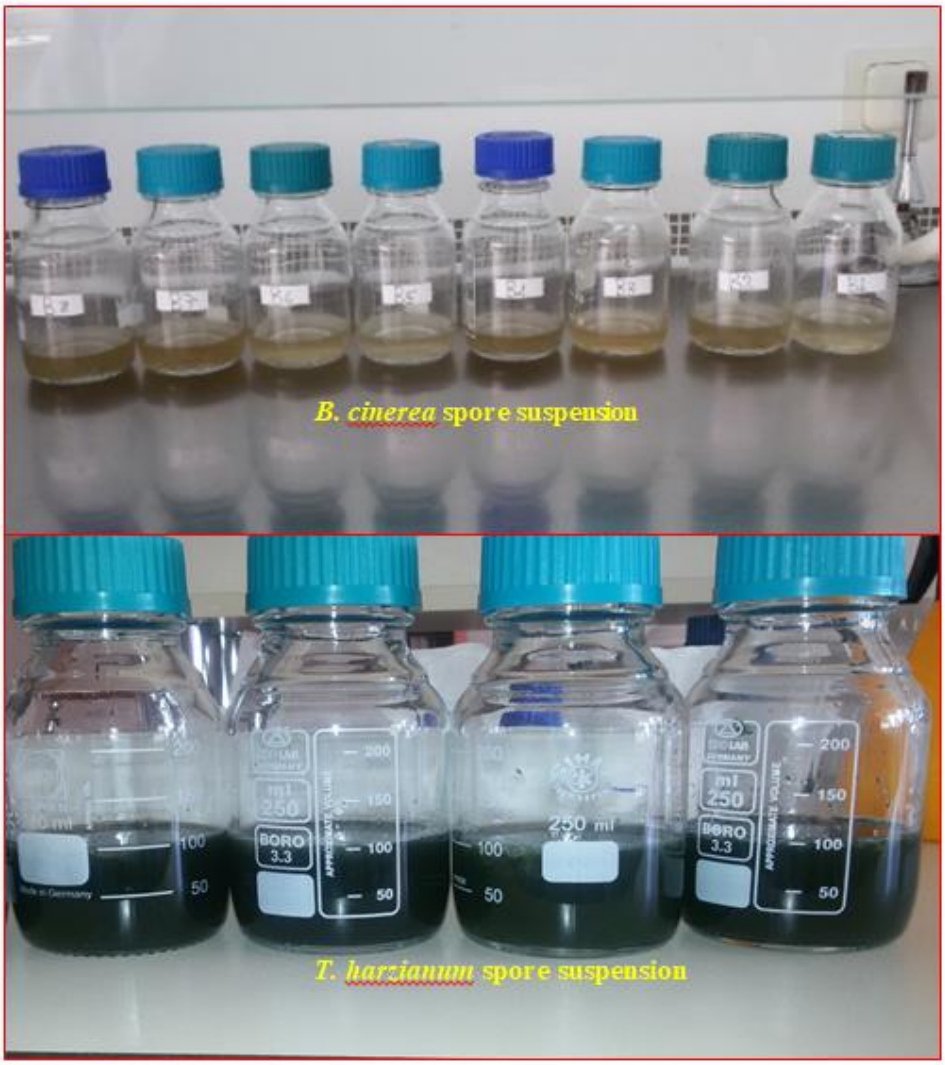

Figure 3.9. Spore suspensions of *B. cinerea* and *T. harzianum* used for antagonistic assessment under glasshouse conditions

After 24 hours of incubation, crops were inoculated with an equal concentration of *B. cinerea*. Pots inoculated with the pathogenic fungus alone were served as the control. Thereafter, inoculated pots were covered with a white polyethylene sheet (Figure 3.9 and 3.10) for 4 days to increase the relative humidity of the environment. Disease severity was rated using a 1-9 scale as described in Table 3.6.

Figure 3.10. Pots covered with polyethylene sheet after inoculation (A), removal of polyethylene sheet 4 days after inoculation (B) under glasshouse conditions

3.2.8.4. Evaluation of Selected T. harzianum against Chocolate Spot under Field Conditions

The field experiment was conducted at Plant Protection Research Station, Faculty of Agriculture, Cukurova University in 2017and 2018. The experiment was laid out in randomized complete block design, replicated three times, and repeated twice. Inter-row and intra-row spacings were 50 and 25 cm, respectively (Figure 3.11). The distance between the blocks was 100 cm, 2 seeds per hill were planted, thinned to one 20 days after planting. The antagonistic *T. harzianum* isolates (T1,

T2, T3, T4, T5, T6, T7, T8, T9 and T10) and as well as mixture of virulent *B. cinerea* isolates (Bc7 and Bc8) were used throughout this experiment.

Figure 3.11. Antagonistic assessment under field conditions; plots covered with polyethylene sheet after inoculation (A), removal of polyethylene sheet 5 days after inoculation (B)

The spore suspension of *T. harzianum* isolates was prepared, 45 old faba bean plants were sprayed with 20 ml/plant at a concentration of 2.5×10^5 spores/ml (Mohammed et al., 1994). After 24 hours of incubation, crops were inoculated with an equal concentration of *B. cinerea*. Plots inoculated with the pathogenic fungus alone were served as the control. Thereafter, inoculated plots were covered with a white polyethylene sheet for 4 days. Agronomic practices (weeding, cultivation, and irrigation) were done as necessary, but no fertilizers were applied. The experiment was repeated twice.

3.2.9. Data Analysis

Analysis of variance (ANOVA) was applied to the colony diameters and radial growth rates of both *B. cinerea* and *T. harzianum* as affected by different temperatures. Colony morphology of *B. cinerea* like number of sclerotia per plate; antagonistic data under laboratory (colony diameter on dual culture), disease severity and area under disease progress curve on detached leaves, glass-house as well as under field conditions were subjected to either statistical analysis software, computer package, version 9.2 (SAS, 2009) or Sigma Stat, version 4. Means were separated using Duncan multiple range tests (DMRT) at the 5 % level of significance.

4. RESULTS AND DISCUSSION

4.1. Colony Growth and Morphological Characterization of *B. cinerea* Isolates

4.1.1. Colony Growth Characteristics of *B. cinerea*

There were variations in colony characteristics among the isolates of *B. cinerea* collected from Adana province, Turkey during 2015/16 and 2016/17. Different growth patterns among the isolates of *B. cinerea* were examined on PDA at 20±2 °C under 12 h dark and 12 h light conditions. They showed radial (A and B), warty (C), compact (D), cottony with a concentric ring (E), radial (F), powdery (G), radial (H). Colony colors were initially dirty white, grayish white and then turned gray to dark gray with age (Figure 4.1).

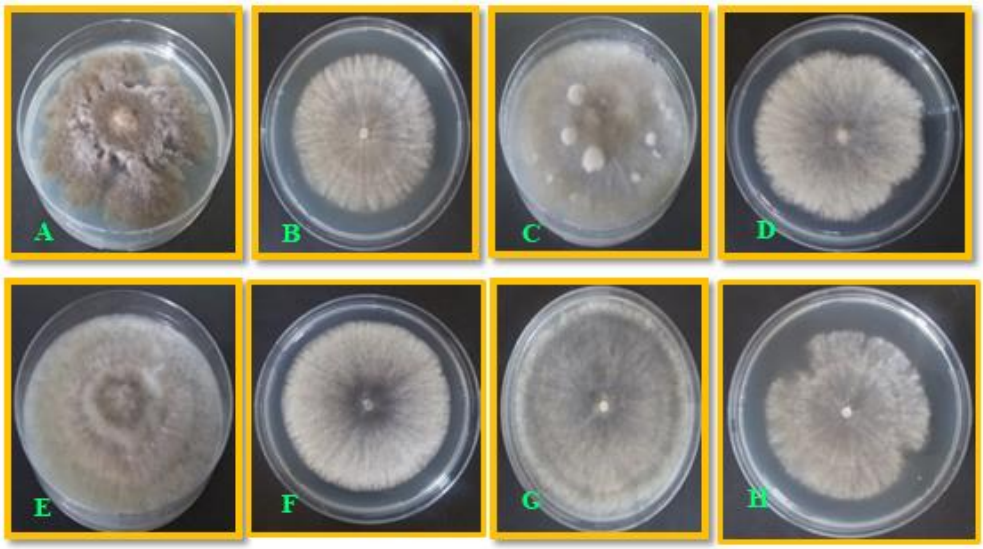

Figure 4.1. Colony growth patterns of *B. cinerea* on Potato Dextrose Agar (PDA); radial (A, B, F and H), warty (C), compact (D), cottony with a concentric ring (E), powdery (G)

Zhang et al. (2010) revealed that *B. cinerea* infecting faba bean was characterized by the development of fluffy, gray-white, villiform aerial mycelia and black sclerotia. The current study also agrees with a study conducted by Hosen et al. (2010) in that the colony characteristics of *B. cinerea* isolates causing chickpea gray mold showed variation. Consequently, they showed cottony white, ashy white, off-white, light ash and greenish white colony color. Moreover, the above authors also revealed the colony texture of the fungus-like fluffy, velvet and effuse on different isolates of the fungus. Moreover, Aktaruzzaman et al. (2017b) also pointed out that the *B. cinerea* colony on the PDA was initially white and then turned gray to dark gray as the colony getting old. According to Miclea et al. (2012), the conidia of *B. cinerea* isolates were differences in abundance, and always produced on the surface of the medium. Some isolates could produce conidia in patches while others all over the plate. The sporulation began from the margin and sometimes from the center. Some isolates could also form the conidia in concentric rings on the plate margins.

4.1.2. Morphological Characterization of *B. cinerea*

In the current study, conidia of *B. cinerea* appeared in clusters, their color was pale brown, non-septate, shapes were elliptical to ovoid, and sizes were 7.0–14 × 6.0–11.5 and 7.0–12.5 × 5.5–9.5 µm (n=50) on Bc6 and Bc8 isolates, respectively (Figure 4.2 and Table 4.1). Conidiophores of the fungus were erected, gray, septate and branched near the top (Figure 4.3). A study conducted by Kwon and Park (2003) revealed that conidial characteristics of *B. cinerea* were single-cell, gray or hyaline, mostly ovoid or ellipsoid and varied in their size.

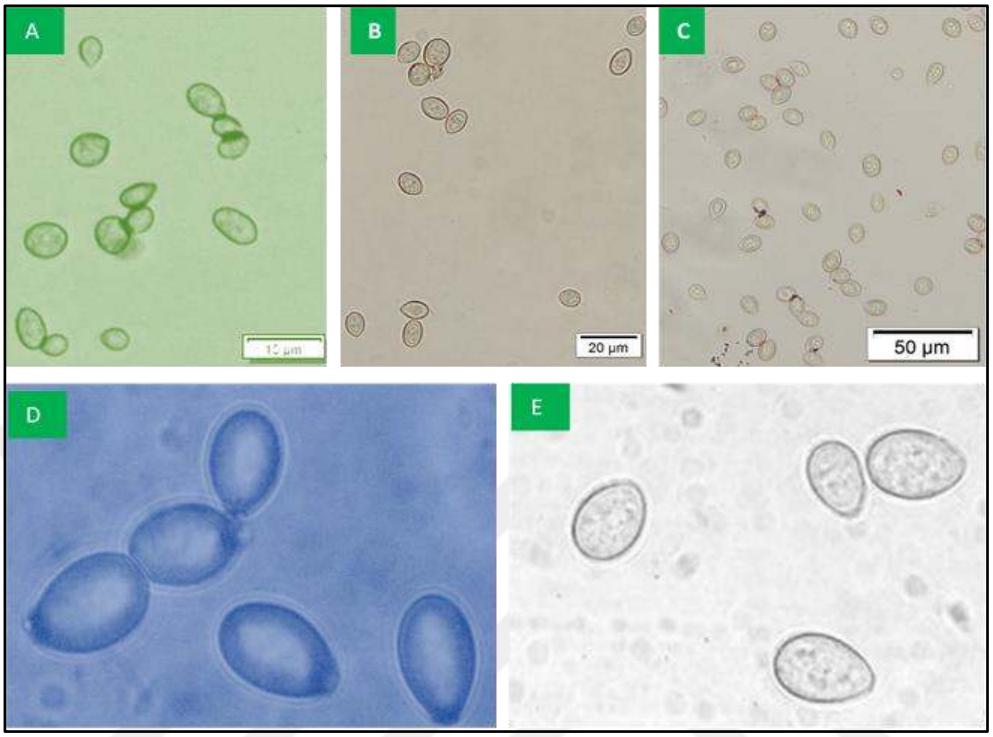

Figure 4.2. Conidial sizes of *B. cinerea* under different objective lenses; conidia (10 µm) (A), conidia (20 µm) (B), conidia (50 µm) (C), conidia (10 µm) under 100× oil immersion (D and E)

Moreover, these researchers had also revealed that conidiophores thickness was produced on PDA, their length and width were different among the isolates, and the sclerotia were produced in the same medium at an optimum temperature of 20 °C. However, the current finding showed that sclerotia were developed well at 17±2 °C. The present study also concurrent with Miclea et al. (2012) in that all conidiophores were straight, monopodial branched towards the apex and septate. The color of conidia was hyaline to pale brown, but becoming dark gray with age, shapes were ellipsoidal to globose, and often unicellular.

Table 4.1. Conidial characteristics of *B. cinerea* isolates, identified from faba bean grown on PDA at 20±2 °C

Treatmen	Color	Shape	[a]Size (µm)	[b]Mean (µm)	L: W ratio
Bc1	Pale brown	Elliptical, Ovoid	6.0–12.5 × 4.5–9.2	10.0 × 6.9	1.45
Bc2	Pale brown	Elliptical, Ovoid	6.0–15.5 × 5.0–9.0	10.7 × 6.6	1.63
Bc3	Pale brown	Elliptical, Ovoid	8.0–13.5 × 5.0–9.5	10.2 × 7.1	1.44
Bc4	Pale brown	Elliptical, Ovoid	7.0–12.5 ×6.0–10.0	9.9 × 7.9	1.26
Bc5	Pale brown	Elliptical, Ovoid	6.0–14.0 ×1.5–9.5	7.9 × 10.2	1.29
Bc6	Pale brown	Elliptical, Ovoid	7.0–14 × 6.0–11.5	11.2 × 9.3	1.20
Bc7	Pale brown	Elliptical, Ovoid	6.5–13.0 × 5.0–8.5	9.5 × 6.6	1.44
Bc8	Pale brown	Elliptical, Ovoid	7.0–12.5 × 5.5–9.5	10.4 × 7.9	1.31
Bc9	Pale brown	Elliptical, Ovoid	7.0–13.0 × 6.0–9.5	9.9 × 7.8	1.26
Bc10	Pale brown	Elliptical, Ovoid	6.0–11.0 × 5.0–10.0	9.7 × 7.9	1.23
Bc11	Pale brown	Elliptical, Ovoid	6.5–13.0 × 6.0–10.0	10.2 × 7.9	1.27
Bc12	Pale brown	Elliptical, Ovoid	6.0–13.0 × 6.0–9.5	9.3 × 7.2	1.29
Bc13	Pale brown	Elliptical, Ovoid	6.0–12.0 × 5.0–9.5	9.3 × 7.2	1.31
Bc14	Pale brown	Elliptical, Ovoid	6.5–12.5 × 5.0–11.0	9.1 × 7.2	1.28
Bc15	Pale brown	Elliptical, Ovoid	6.5–12.5 × 5.0–10.0	9.4 × 7.4	1.27
Bc16	Pale brown	Elliptical, Ovoid	5.5–11.0 × 5.0–8.5	8.7 × 6.4	1.37
Bc17	Pale brown	Elliptical, Ovoid	7.0–13.0 × 5.5–10.0	9.4 × 7.3	1.29
Bc18	Pale brown	Elliptical, Ovoid	5.5–12.5 × 5.0–8.5	9.2 × 6.6	1.39

[a]Conidial size: Length ((min-max) × width (min-max)), [b]Conidial mean: length of mean × width (breadth) of mean

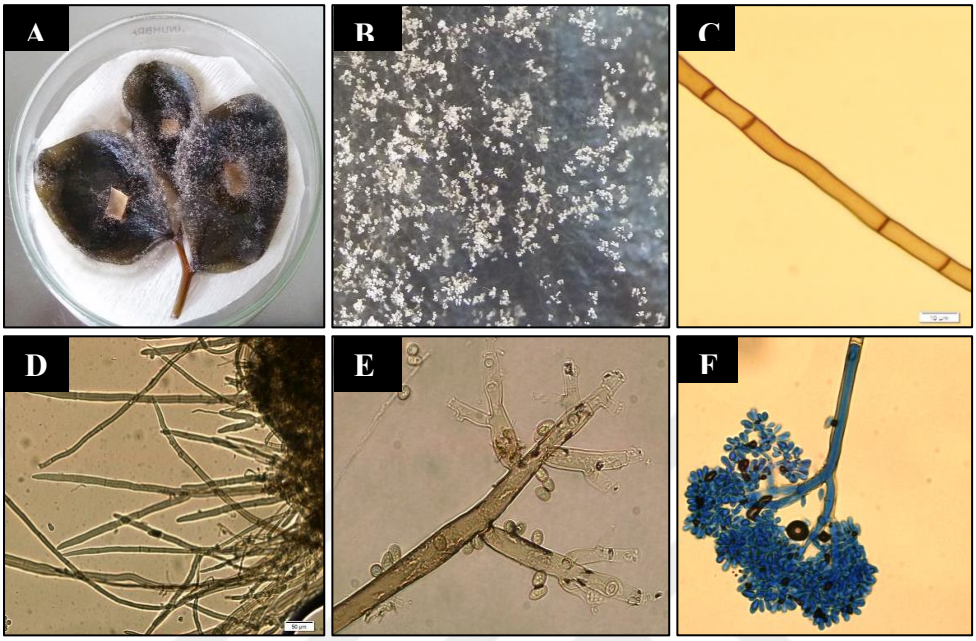

Figure 4.3. Morphological characteristics of *B. cinerea* conidiophores; growth of conidiophores on detached faba bean leaves (A), conidiophores view under stereomicroscope (B), septation of conidiophore (C), development of conidiophores from mycelia (D), conidiophore with few conidia (E), conidiophores stained with lactophenol blue (F)

After 20 days of incubation, the fungal isolates formed different sclerotial growth patterns and arrangements. For instance: Bc1 and Bc3 exhibited large size sclerotia, grow all over the medium, and placed irregularly; Bc4 very few in number and large sizes of sclerotia; Bc2, Bc7 and Bc8 produced large sclerotia that arranged regularly (circular) near the edges of Petri plate; Bc9 also exhibited large to small size of sclerotia, arranged irregularly near the edge of the Petri dish; Bc10 produced few numbers of sclerotia, that were small in size and arranged at the edge of Petri plate; Bc11 produced large sclerotia and arranged in a circle; Bc14 formed medium size sclerotia and arranged irregularly at the edge of the Petri plate (Figure 4.4).

Moreover, these isolates exhibited black, hard, and irregular to flat shapes sclerotia as well as they showed variation in distribution and abundance. Radial growth rates of mycelium and a number of sclerotium per dishes showed significant ($P \leq 0.05$) differences among the treatments (Table 4.1). The maximum and minimum sclerotial sizes of 3.0–6.0×2.0–4.5 and 1.5–4.0×1.3–3 mm (n=30) were measured on Bc8 and Bc12 isolates, respectively. Moreover, the highest (113.3) and lowest (33) number of sclerotia per dish were counted on Bc9 and Bc2 isolates, respectively. As well as the maximum radial growth rate (13.5mm/d) was calculated on Bc17 while the minimum (6.5 mm/d) was from Bc3 (Table 4.2). Aktaruzzaman et al. (2017b) also revealed that *B. cinerea* could produce irregular, hard and black sclerotia. The current study also in line with the one carried out by Abdel Wahab (2015) in that *B. cinerea* isolated from different host crops that showed variation in mycelial growth and sclerotia production.

The results of current resembled to a study conducted by Xun-yi (1989) in that there was morphological variation among *B. fabae* isolates; accordingly, some of them produced few sclerotia with excessive growth of mycelium. On the other hand, some isolates produced abundant sclerotia with minimal growth of mycelium, and the others also showed moderate production of sclerotia and mycelium. Similarly, the study conducted by Hosen et al. (2010) in agreement with the present study in that all *B. cinerea* isolates, causing chickpea gray mold, produced black color sclerotia, some of them produced very few in number, arranged irregularly in peripheral regions of Petri plates. Hutson and Mansfield (1980) also make this point clear in that there were morphological variation and aggressiveness between isolates of *B. fabae* that causing faba bean chocolate spot.

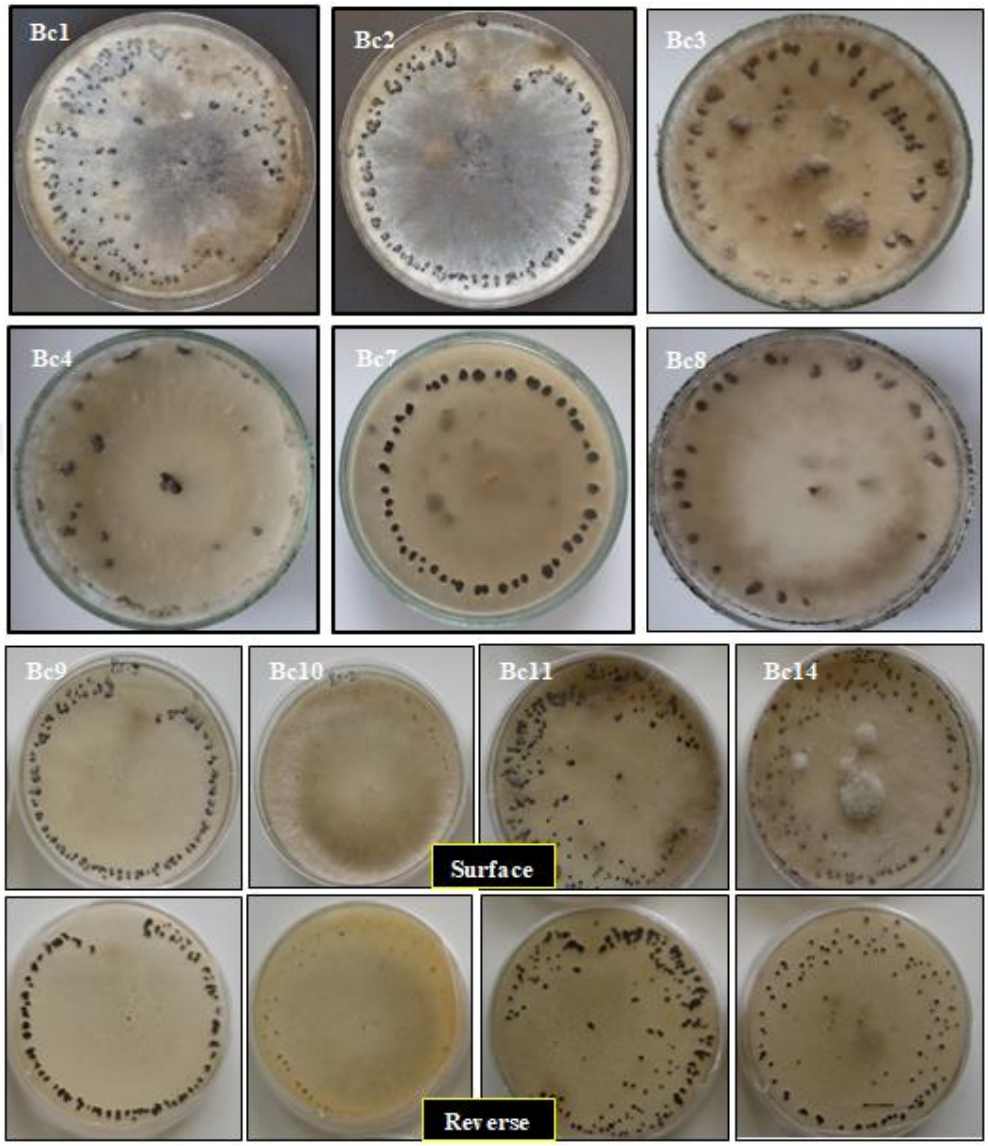

Figure 4.4. The distribution patterns of the sclerotia produced by *B. cinerea* 20 days after the inoculation on PDA at 17±2 °C under dark conditions

Table 4.2. Mycelial growth and sclerotial characteristics of isolates of *B. cinerea*

Isolates	RGR[a] (mm/d)	Sclerotia[b]					
		Color	Shape	No./dish	Size (mm)[b]	Mean[c] (mm)	L/W
Bc1	10.1 bc	Black	Irregular/flat	38.3 cd	2.0–7.0 ×1.5–6.4	4.1 ×3.4	1.20
Bc2	9.8 c	Black	Irregular/flat	33.0 d	2.0–9.5 × 1.8–6.0	5.1 × 3.2	1.58
Bc3	6.5 d	Black	Irregular/flat	37.3 cd	2.0–5.5 × 1.5–4.0	3.8 × 2.8	1.38
Bc4	12.7 ab	Black	Irregular/flat	47.7 cd	2.8–5.3 × 2.3–4.0	4.0 × 2.9	1.37
Bc5	9.9 bc	NA	NA	NA	NA	NA	NA
Bc6	10.8 abc	Black	Irregular/flat	48.7 cd	2.5–9.5 × 1.8–5.3	4.9 × 2.9	1.69
Bc7	11.7 abc	Black	Irregular/flat	93.0 abc	2.0–8.0 × 2.0–4.5	4.4 × 2.8	1.59
Bc8	12.0 abc	Black	Irregular/flat	59.7 abcd	3.0–6.0 × 2.0–4.5	4.2 × 3.1	1.38
Bc9	10.8 abc	Black	Irregular/flat	113.3 a	2.0–11.5 × 1.7–5.0	3.6 × 2.3	1.55
Bc10	10.5 bc	Black	Irregular/flat	85.0 abcd	1.8–5.0 × 1.8–3.0	2.4 × 2.0	1.19
Bc11	10.6 bc	Black	Irregular/flat	75.0 abcd	2.1–8.0 × 1.5–3.2	4.0 × 2.5	1.60
Bc12	10.9 abc	Black	Irregular/flat	81.3 abcd	1.5–4.0 × 1.3–3.0	2.4 × 1.9	1.25
Bc13	10.4 bc	Black	Irregular/flat	107.7 ab	2.0–5.0 × 1.5–3.0	2.8 × 2.1	1.30
Bc14	11.8 abc	Black	Irregular/flat	83.3 abcd	2.0–5.0 × 2.0–4.0	3.2 × 2.4	1.35
Bc15	11.5 abc	Black	Irregular/flat	60.0 abcd	1.5–5.5 × 1.5–4.0	3.0 × 2.2	1.35
Bc16	11.8 abc	Black	Irregular/flat	49.7 cd	1.5–6.0 × 1.5–3.5	3.3 × 2.0	1.64
Bc17	13.5 a	Black	Irregular/flat	53.3 bcd	2.0–7.0 × 1.0–3.5	3.7 × 2.2	1.65
Bc18	12.5 abc	Black	Irregular/flat	61.3 abcd	1.5–6.0 × 1.0–4.0	3.4 × 2.3	1.48

[a]Radial growth rate calculated after incubating at 20 °C for 2 days on PDA, [b]Sclerotial size: Length ((min-max) × width (min-max)), [c]Sclerotial mean: length of the mean × width (breadth) of mean, NA: not available

4.2. Molecular Identification of *Botrytis* and *Trichoderma* Species

4.2.1. Agarose Gel Electrophoresis Study

The ITS5/ITS4, as well as β-tubulin1/β-tubulin2 regions for both *B. cinerea* and *B. fabae*, were amplified, and the product sizes of 500 bp were obtained for *B. cinerea* specie (Figure 4.5). For *B. fabae*, 600 and 500 bp sizes were obtained for β-tubulin and ITS gene regions, respectively (Figure 4.6). Similarly, ITS5-ITS4 and translation elongation factor one alpha (TEF1α) gene regions for 10 isolates of *T. harzianum* isolates were amplified, and 700 and 1000 bp DNA fragments obtained for ITS and TEF1α, respectively (Figure 4.7).

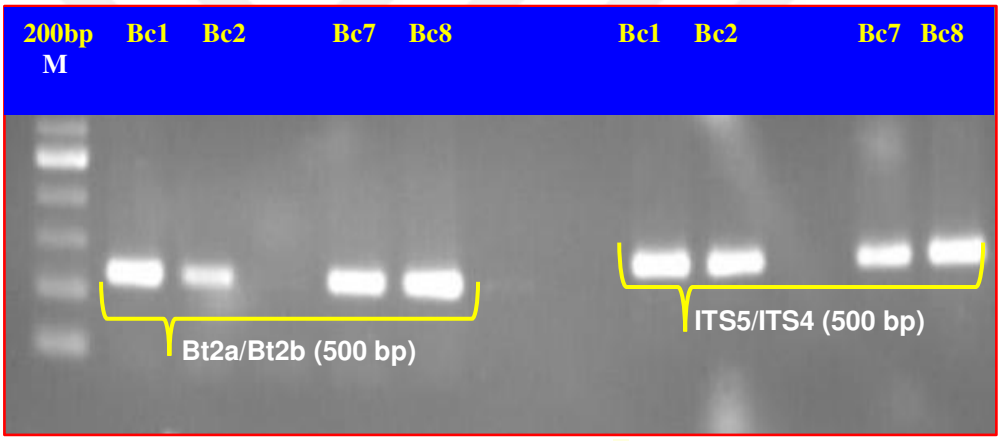

Figure 4.5. Electrophoresis products obtained with β-tubulin and ITS gene regions for the *B. cinerea* (Bc1, Bc2, Bc7 and Bc8) isolates during the optimized PCR reaction

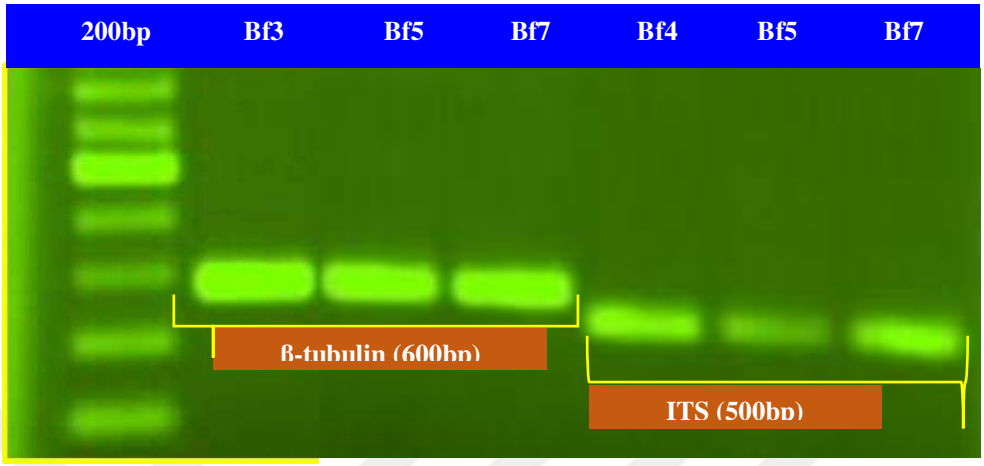

Figure 4.6. Electrophoresis products obtained with β-tubulin and ITS gene regions for the *B. fabae* (Bf3, Bf4, Bf5 and Bf7) isolates during the optimized PCR reaction

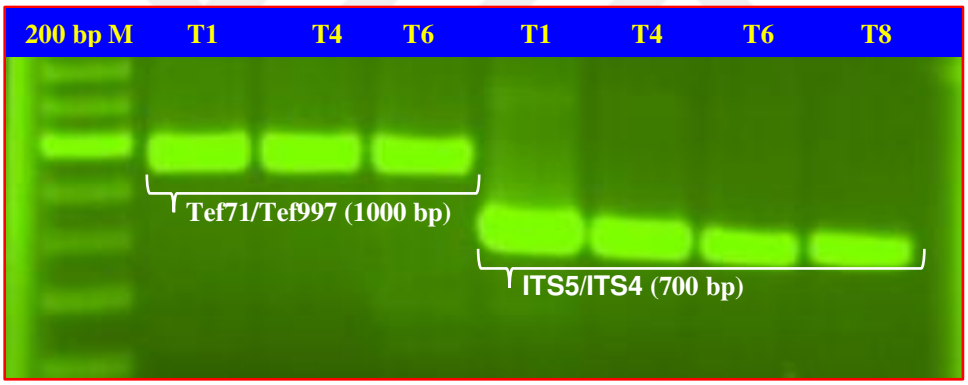

Figure 4.7. Electrophoresis products obtained with *tef1*-α and ITS gene regions for *T. harzianum* (T1, T4, T6 and T8) isolates during the optimized PCR reaction

4.2.2. Registration of Selected Isolates of *Botrytis* and *Trichoderma* spp. in GenBank

The accession number of a total of 8 *Botrytis cinerea* and 8 *Botrytis fabae*, identified on faba bean in Adana (Turkey) and Ethiopia, respectively, as well as 8 isolates of *Trichoderma* strains, were registered in GenBank. They were presented in Table 4.3 and 4.4.

Table 4.3. Accession numbers of isolates of *Botrytis* spp. causing faba bean chocolate spot (https://www.ncbi.nlm.nih.gov) in the current study

Pathogen Species	Isolate No. ITS/ β-tubulin	Origin and Year	GenBank Accession No.	
			ITS	β-tubulin
B. cinerea	ASF-Bc1	Turkey, Adana, 2016	MH208266	MH208274
	ASF-Bc2	Turkey, Adana, 2016	MH208267	MH208275
	ASF-Bc3	Turkey, Adana, 2016	MH208268	MH208276
	ASF-Bc4	Turkey, Adana, 2016	MH208269	MH208277
	ASF-Bc5	Turkey, Adana, 2016	MH208270	MH208278
	ASF-Bc6	Turkey, Adana, 2016	MH208271	MH208279
	ASF-Bc7	Turkey, Adana, 2016	MH208272	MH208280
	ASF-Bc8	Turkey, Adana, 2016	MH208273	MH208281
B. fabae	EHF-Bf1	Ethiopia, Haramaya, 2014	MK217904	MH208282
	EHF-Bf2	Ethiopia, Haramaya, 2014	MK217905	MH208283
	EHF-Bf3	Ethiopia, Haramaya, 2014	MK217906	MH208284
	EHF-Bf4	Ethiopia, Haramaya, 2014	MK217907	MH208285
	EHF-Bf5	Ethiopia, Haramaya, 2014	MK217908	MH208286
	EHF-Bf6	Ethiopia, Haramaya, 2014	MK217909	MH208287
	EHF-Bf7	Ethiopia, Haramaya, 2014	MK217910	MH208288
	EHF-Bf8	Ethiopia, Haramaya, 2014	MK217911	MH208289

Table 4.4. Accession numbers of *T. harzianum* isolates from aerial parts of faba bean (https://www.ncbi.nlm.nih.gov) during 2016 and 2017

Species	Isolate No. ITS/Tef1–α	Substrates	GenBank Accession No.	
			ITS	Tef1–α
T. hazianum	ASF-T1	*Vicia faba* leaf	KY783938	MH208248
	ASF-T2	*V. faba* leaf	MH208242	MH208249
	ASF-T3	*V. faba* leaf	MH208243	MH208250
	ASF-T4	*V. faba* stem	KY783939	MH208251
	MYF-T7	*V. faba* leaf	MH208244	MH208252
	MYF-T8	*V. faba* leaf	MH208245	MH208253
	ASF-T9	*V. faba* leaf	MH208246	MH208254
	ASF-10	*V. faba* stem	MH208247	MH208255

4.2.3. Phylogenetic Analysis

The BLAST search of NCBI followed by phylogenetic sequence analysis of β-tubulin gene region showed that all *Botrytis* isolates (MH208282, MH208283, MH208284, MH208285, MH208286, MH208287, MH208288 and MH208289) identified from faba bean leaves in Ethiopia had 99% sequence identity with

Botrytis fabae retrieved from GenBank (with the accession number of KR054109 identified in Germany) while all isolates of *Botrytis* spp. (MH208274, MH208275, MH208276, MH208277, MH208278, MH208279, MH208280 and MH208281), identified from the same host (faba bean) in Adana, Turkey, had 95-100% identity with *Botrytis cinerea* (GenBank accession number MG949129, MG949128, MG949127, MG949126, MG949125, CP009805, KY775145, KM249110 and CP009805), that were identified in China, Netherlands, Korea and USA (Figure 4.8). In this study, sequence analysis of the ITS gene region showed homogeneous (the tree was not shown). Therefore, this primer is not an ideal candidate for *Botrytis* species identification.

Figure 4.8. Phylogenetic relationship of *Botrytis* spp. based on Maximum Likelihood (ML) method analysis of Bt2a-Bt2b sequences. Numbers on the branch nodes represent bootstrap support values (%) based on1000 replications. *Monilinia polystroma* was included as an outgroup. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 367 positions in the final dataset.

According to ITS sequences, all the isolates of *Trichoderma* strains identified from both faba bean (*Vicia faba*) and field pea (*Pisum sativum*) (KY783938, MH208242, MH208243, KY783939, MH208244, MH208245, MH208246 and MH208247) had 99-100% sequence identity with *Trichoderma harzianum* with the accession number in a GenBank (Tunisia isolate: EU595033), (China isolates: MG707197 and MG707200), (India isolate: MH333257), and (Thailand isolates: JN116598, JN116680 and JN116715) (Figure 4.9 and 4.11). Based on tef1-α sequences, isolates of *Trichoderma* identified in this study (MH208248, MH208249, MH208250, MH208251, MH208252, MH208253, MH208254 and MH208255) had 92 to 99% sequence identity with reference strain of *Trichoderma harzianum* (anamorph stage), *Hypocrea lixii* (teleomorph stage) (EF191330), AY605764, EF191332, KJ871174, KJ871186, EF191322, AY605779, EF191329, AY605783, KU738448, HG931204, FJ763166, FJ763177 and FJ763164, EF191333), that were identified in India, Thailand, Tunisia and China (Figure 4.10 and 4.11).

According to Zarrin et al. (2016), internal transcribed spacer 1 and 2 are employed for the identification of several fungal species. Because they have a certain advantage of it is a good target for detection. Apart from this, this region is basically conserved within several species of fungi. In the genome of fungi, this region is basically present as multiple copies, and produce a sufficient taxonomic resolution for the numerous fungi. Currently, the availability of the large number of sequences from ITS locus in the GenBank makes this region more vital and enable the comparison of the obtained sequences.

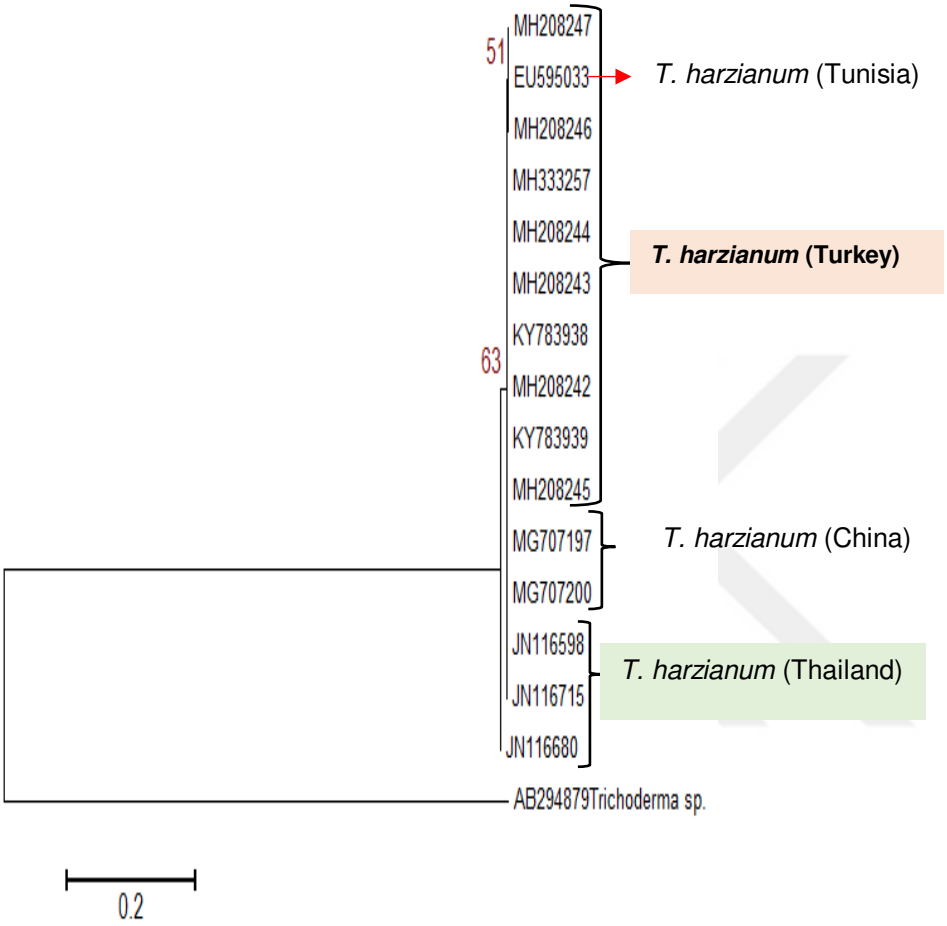

Figure 4.9. Phylogenetic relationship of *Trichoderma* spp. based on Maximum Likelihood (ML) method analysis of ITS sequence. Numbers on the branch nodes represent bootstrap support values (%) based on1000 replications. *Trichoderma* sp. was included as an outgroup. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 541 positions in the final dataset.

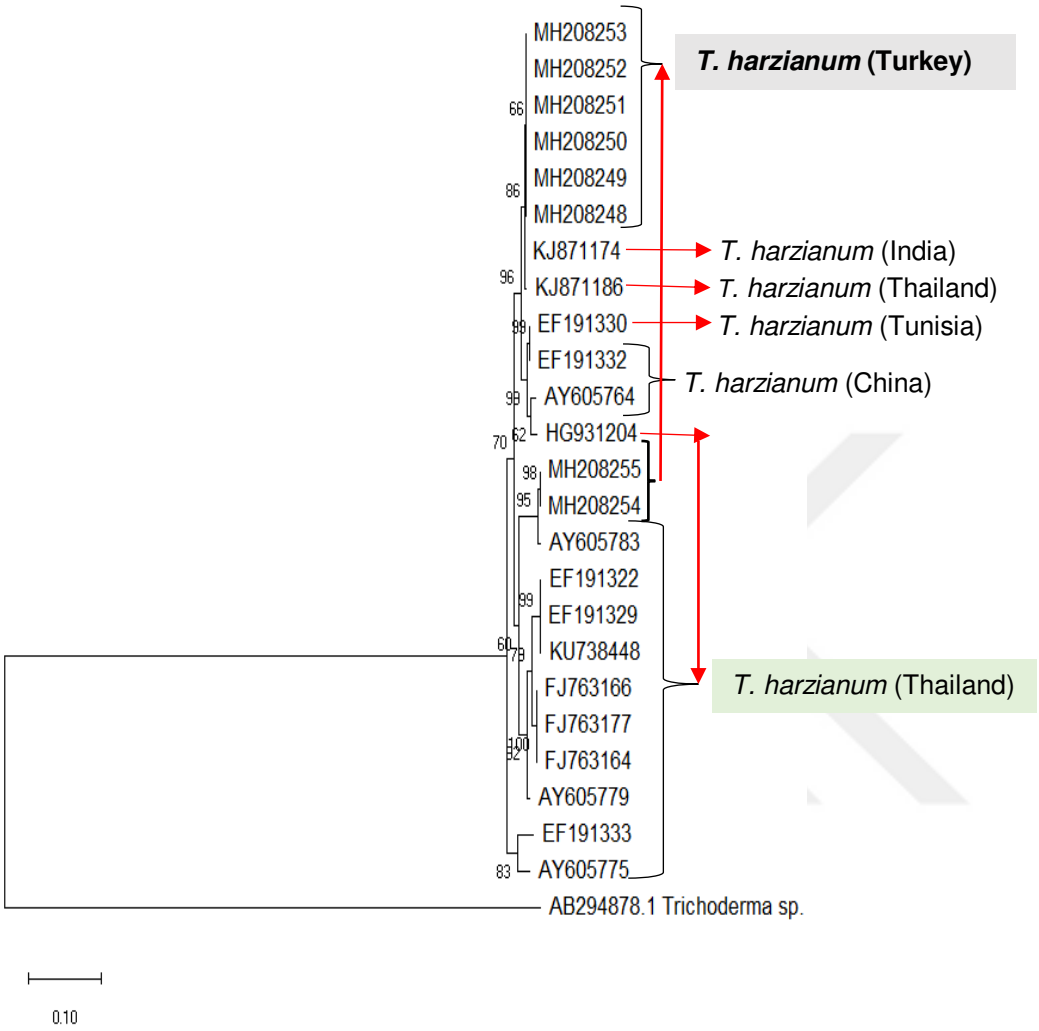

Figure 4.10. Phylogenetic relationship of *Trichoderma* spp. based on Maximum Likelihood (ML) method analysis of tef1-α sequences. Numbers on the branch nodes represent bootstrap support values (%) based on1000 replications. *Trichoderma* sp. was included as an outgroup. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 765 positions in the final dataset.

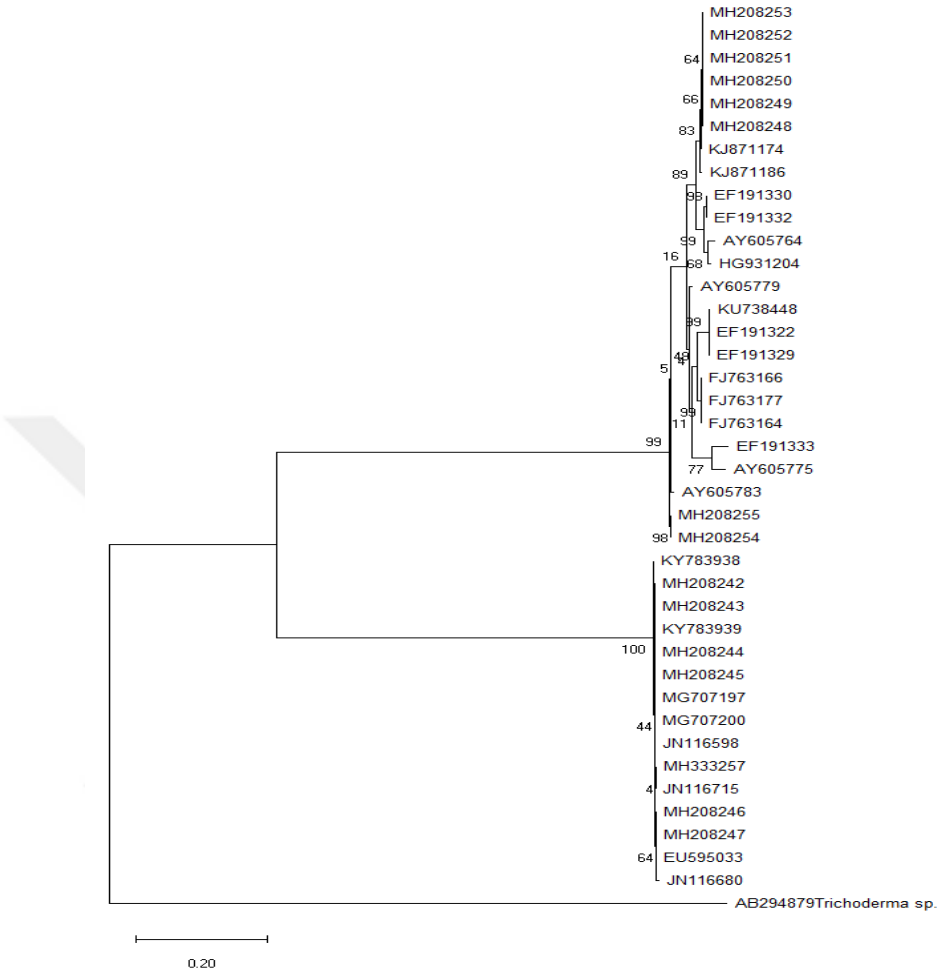

Figure 4.11. Phylogenetic relationship of *Trichoderma* spp. based on Maximum Likelihood (ML) method analysis of combined ITS and tef1-α sequences. *Trichoderma* sp. was included as an outgroup. Numbers on the branch nodes represent bootstrap support values (%) based on1000 replications. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 581 positions in the final dataset.

4.3. Effect of Different Temperature on Mycelial Growth of *B. cinerea* and *T. harzianum*

4.3.1. Effect on *B. cinerea* Growth

The colony diameter and radial growth rate of the fungus on potato dextrose agar (PDA) were significantly ($P \leq 0.05$) affected by different temperatures as shown in Table 4.5 and Figure 4.12. The mycelial radial growths of pathogen increased with each temperature up to 20 °C and declined thereafter. The mycelium starts growing 7 and 3 days after inoculation at 5 and 10 °C, respectively. Nevertheless, the fungus completely covers the Petri plate within 6 days at 15 and 20 °C. Similarly, the optimum radial growth for all isolates was obtained at 20 °C and followed by 15 °C while no growths were observed at 0 and 35 °C. The present study in agreement with several investigators in that growth and development of *Botrytis* spp. are highly affected by temperature. Accordingly, the optimum temperature for mycelial growth and development of the fungi isolated from fig fruit was 18 °C. Maximum and minimum temperatures for the *Botrytis cinerea* development was 38 and 0 °C, respectively (Czaja et al. (2016). However, in the current study, no growth was observed at 0 and 35 °C for this fungus. Our study also congruent with a study conducted by Garrido and Sônego (2005) in that growth and development of *Botrytis* spp. were favored by temperatures ranging from 18 to 23 °C. Similar to the present study, the optimum temperature for the *B. cinerea* mycelial radial growth was recorded at 20 °C, and the growth rate increased up to the temperature of 20 °C then it reduced gradually up to 30 °C and at 35 °C (Hosen et al., 2010). A study conducted by Abdel Wahab (2015) showed that different *B. cinerea* isolates grew rapidly on PDA at 23˚C with average mycelial radial growth rate (MGR) ranging from 20 to 36 mm/d, and many isolates developed and covered the entire dish within three to four days. Moreover, these isolates able to produce aerial mycelia, conidia, sclerotia within 15 days old cultures. Harrison (1980) also reported that the chocolate spot disseminates by airborne conidia from infected crops to another temperatures range of 15-20 °C and

relative humidity above 70%. Similarly, in the present study, we noticed that maximum mycelial growths were observed at 20 °C and 15 °C. Accordingly, we suppose that chocolate spot can cause disease symptom at the optimum temperature of 15-20 °C, with a maximum 30 °C and minimum of 4 °C. Water films and light intensity have no impact on symptom development (Harrison, 1980), but light can induce sporulation (Harrison and Heilbronn, 1988).

Table 4.5. Effect of different temperature on colony diameter of *B. cinerea* isolates, 3 days after mycelia start growing

Treatment	Colony diameter (mm)[a]					
	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C
Bc1	20.5±1.8cd	41.8±1.0a	49.5±1a	55.3±5.0ab	47.5±1.0a	34.8±3.5a
Bc2	17.8±1.0d	29.3±8.5b	42.5±5.2b	48.0±2.6b	42.2±1.2b	35.3±1.9a
Bc3	21.5±1.3cd	18.2±7c	48.7±2.3ab	36.8±12.4c	33.7±1.9c	32.2±1.9ab
Bc4	22.8±3.3bc	46.0±1.3a	49.7±0.3a	57.8±4.5ab	26.2±2.1ef	23.8±0.8d
Bc5	21.2±1.0cd	39.5±0.5a	48.0±2.6ab	56.0±0.0ab	22.7±2.4f	8.7±1.3f
Bc6	21.7±3.4cd	40.5±4.3a	47.3±5.0ab	54.2±2.9ab	28.0±2.6de	19.3±3.0e
Bc7	27.2±0.3a	39.3±4.0a	53.5±4.8a	60.5±2.6a	30.7±3.0cd	29.3±2.5bc
Bc8	26.0±3.0ab	41.0±1.3a	53.8±1.5a	63.8±0.8a	29.8±1.5de	26.7±0.8cd
Mean	22.3±3.4ab	36.9±9.3	49.1±4.4	54.1±9.1	32.6±8.2	26.3±8.8
CV (%)	9.9	12.1	6.9	9.8	6.3	8.2
Sign.	0.002	<0.0001	0.02	0.004	<0.0001	<0.0001

[a] colony diameter was calculated after 3 days incubation, Mean value ± standard deviation (x ± S.D), means followed by the same letters are not significant difference according to Duncan multiple range tests (DMRT), Sign. = significance level

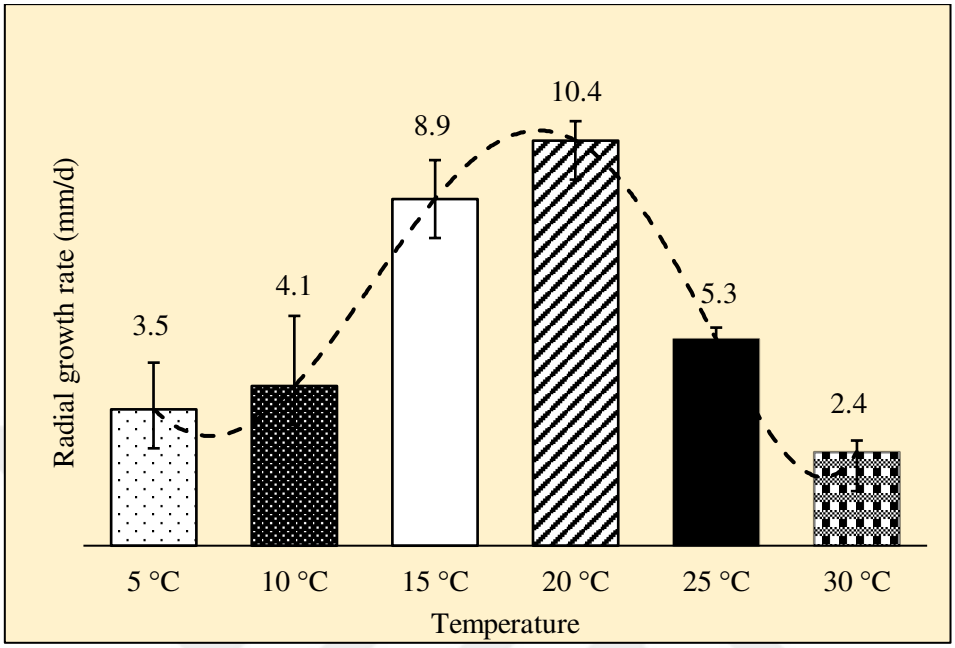

Figure 4.12. The radial growth rate of *B. cinerea* at different temperatures (Mean of 8 isolates)

4.3.2. Effect different Temperature on Growth of *T. harzianum* Isolates

The effect of temperature on the colony diameter and radial growth rate of *T. harzianum* on the PDA was presented in Table 4.6 and Figure 4.13. There was a highly significant ($P \leq 0.0001$) difference between the treatments. The highest mycelial diameter of fungus was recorded at 30 °C and followed by 25 and 20 °C, but the lowest was scored at 10 and 15 °C, and no growth was observed at 5 and 40 °C. Similarly, the highest RGR was calculated for all isolates at 30°C and followed by 25°C. While the lowest RGR was scored at 10 and 35 °C, respectively. There was no colony growth at 5 and 40 °C. Therefore, this study confirmed that an optimum temperature for the mycelial diameter and radial growth rate of *Trichoderma* spp. were 30 °C even though there was variation among the isolates. The present study in agreement with several studies reporting that *Trichoderma* species, including *T. harzianum*, grow best in the temperature range of 25 to 30 °C. Similarly, *Trichoderma virens, Trichoderma viride* and *Trichoderma hamatum*

grew faster at the temperature between 25–30 ºC while at 40 ºC, the fungi were growing slowly (Chennappa et al., 2017). While Hajieghrari et al. (2008) reported that the highest mycelial growth of *T. harzianum* and *T. hamatum* were recorded at a temperature of 25 ºC whereas the mycelial growth of *T. virens* was high at 30 ºC.

As reported by Mukherjee and Raghu (1997), the *Trichoderma* species could not kill the pathogen under *in vitro* condition at temperatures beyond 30 °C. A similar result was also reported by Maurya et al. (2017) that the *T. viride* continued growing until 30˚C and afterward, the growth started decreasing. But, the maximum growth was obtained at 25 to 30 °C. It might be concluded that there was high variation among isolates of *B. cinerea* and *Trichoderma harzianum* against different temperatures.

Table 4.6. Effect of different temperature on colony diameter *T. harzianum* isolates, 3 days after mycelia start growing

Treatment	Colony diameter (CD) (mm) (Mean ± S.d)					
	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C
T1	14.0±6.7cd	20.8±0.8g	57.3±1.6def	81.5±1.5c	85.5±0.5a	34.5±0.9ab
T2	12.2±2.8cd	32.8±1.9ab	54.8±2.5f	82.8±1.6abc	85.8±0.3a	30.3±0.3c
T3	15.7±4.8cd	22.7±4.6fg	67.7±0.7a	85.0±0.0a	86.0±0.0a	34.2±3.1b
T4	12.8±2.4cd	26.5±2.6def	65.5±2.0ab	84.0±0.5ab	86.0±0.0a	34.5±0.9ab
T5	18.0±4.1abc	22.7±1.4fg	62.2±2.4bcd	83.8±0.6ab	86.0±0.0a	34.8±0.8ab
T6	14.5±7.5bcd	36.0±4.8a	60.0±2.3cde	83.8±1.0ab	86.0±0.0a	32.7±0.3b
T7	2.2 ±3.8ef	29.7±2.0bcd	56.0±6.1ef	81.3±1.3c	83.8±1.2b	19.7±1.0e
T8	0.0±0.0f	27.8±2.0cde	61.7±1.7bcd	82.3±1.5bc	86.0±0.0a	15.0±1.5f
T9	8.8±1.3de	31.3±1.3bc	49.7±3.8g	78.5±1.3d	86.0±0.0a	22.2±0.2d
T10	1.5±2.6ef	30.2±1.3bcd	63.2±2.1abc	83.3±1.3abc	86.0±0.0a	15.2±0.6f
T11	24.7±6.0a	20.3±0.8g	54.3±1.2fg	79.0±1.3d	86.0±0.0a	36.7±1.6a
T12	22.2±0.6ab	24.7±2.5efg	54.0±1.3fg	77.8±0.8d	86.0±0.0a	9.7±1.0g
Mean	12.2±8.4	27.1±5.3	58.8±5.6	81.9±2.5	85.8±0.7	26.6±9.4
CV (%)	24	9.3	4.6	1.4	0.4	4.7
Sign.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean not followed by the same letter differ significantly from one another (P=0.05) according to the Duncan multiple range tests, CV: coefficient of variation; Sign. = significance level, S.D= Standard deviation

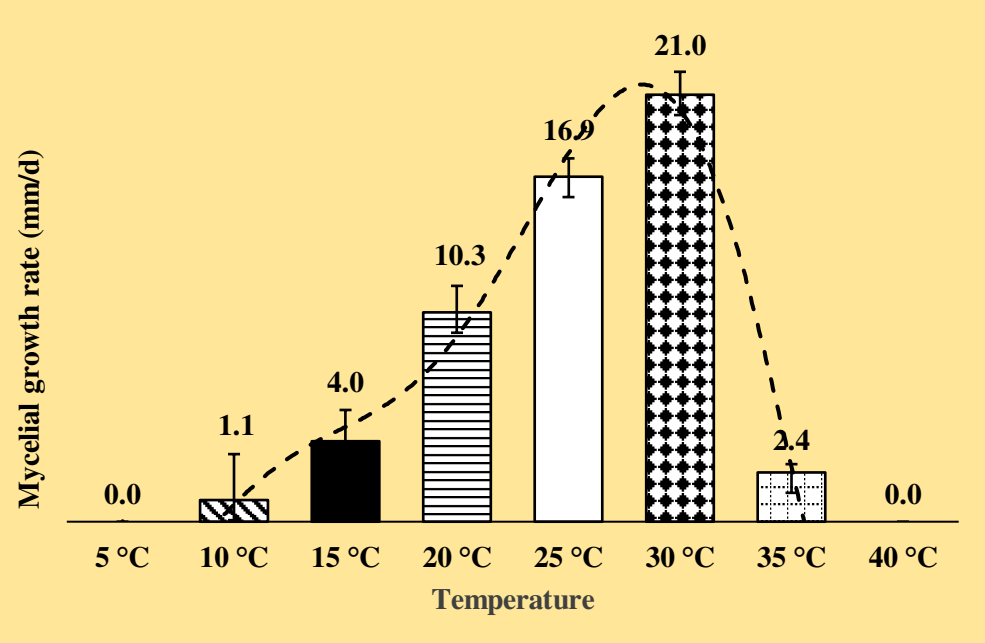

Figure 4.13. Effect of temperature on radial growth rate (means of 12 isolates) of *T. harzianum*, error bars=mean ± Standard deviation

4.4. Pathogenicity Test

4.4.1. On Detached Faba Bean Leaves

Pathogenicity of the isolates on the faba bean leaflets was significant (P≤0.001) different. Isolates of *B. cinerea* developed necrosis symptom on faba bean leaflets 3 days after inoculation (incubation period) at 20±2 °C in the dark and gradually increased, with the days and the leaves were completely covered with the disease symptoms 7 days after inoculation. Accordingly, the maximum lesion length and width were scored on Bc7 (54.3 mm) and Bc8 (56.3 mm), 7 days after inoculation. While the minimum was recorded on Bc6 isolate. Similarly, the highest area under disease progress curves for lesion length was scored on Bc7 (104.2mm–days) and Bc8 (103.3mm–days), as well as the highest AUDPC (71.3 and 75.3mm- days) for lesion widths, were scored on these isolates, respectively (Figure 4.14 and Table 4.7), but control leaflets inoculated with the agar plugs,

which were free of the fungus, remained symptomless. Therefore, the present study pointed out that there was virulence variability among the isolates. The results of current study is in agreement with those of Zhang et al. (2010) reporting that faba bean leaves inoculated with agar plugs containing *B. cinerea* showed the maximum lesion size as compared with the other two *Botrytis* species, *B. fabae* and *B. fabiopsis*.

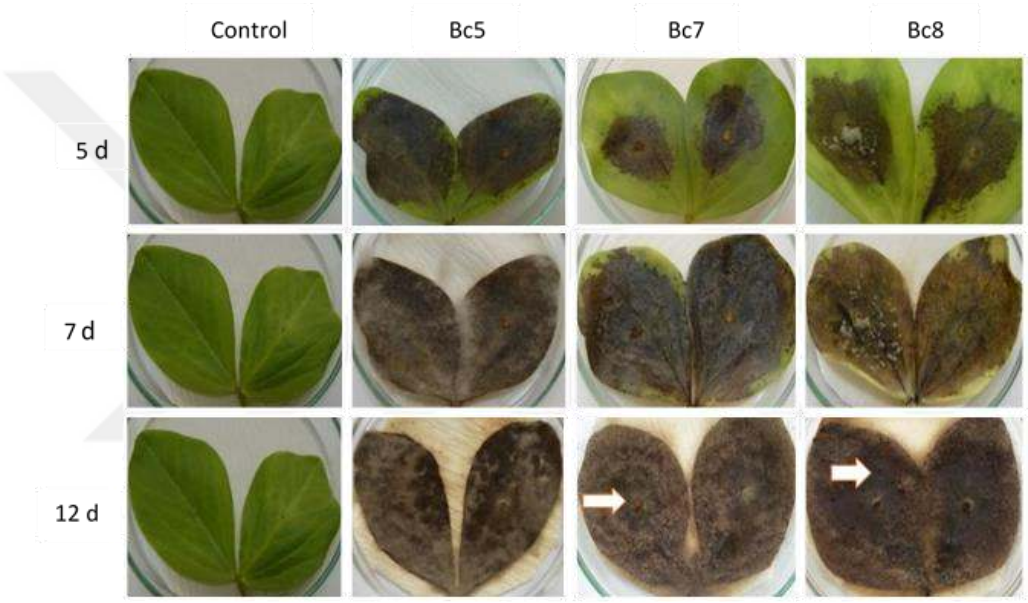

Figure 4.14. Virulence of *B. cinerea* isolates (Bc5, Bc7, and Bc8) on detached faba bean leaves and progress of the disease as inoculated with 3 days old mycelial plugs (4 mm diameter) (20 ±2 °C)

Table 4.7. Lesion size, the area under disease progress curve and disease incidence on leaflets of faba bean inoculated with mycelia of *B. cinerea* isolates

Treatment	5 DAI		7 DAI		AUDPC (mm-days)		Incidence %
	L. length (mm)	L. width (mm)	L. length (mm)	L. width (mm)	Lesion length	Lesion width	
Bc1	46.0(1.67)a	26.5(1.44)a	53.8(1.74)a	29.0(1.49)b	102.3(2.01)a	55.5(1.75)b	100.0
Bc2	38.0(1.59)a	28.5(1.47)a	42.3(1.64)a	32.0(1.52)ab	80.3(1.91)a	60.5(1.79)ab	100.0
Bc3	40.3(1.61)a	27.2(1.45)a	45.0(1.66)a	31.7(1.51)ab	85.3(1.93)a	58.8(1.78)ab	100.0
Bc4	25.5(1.42)b	16.3(1.23)b	29.2(1.48)b	19.0(1.29)c	54.7(1.74)b	35.3(1.56)c	100.0
Bc5	45.7(1.67)a	31.2(1.50)a	51.7(1.72)a	36.0(1.57)ab	97.3(1.99)a	67.2(1.83)ab	100.0
Bc6	21.5(1.35)b	14.3(1.18)b	24.2(1.39)b	16.2(1.23)c	45.7(1.67)b	30.5(1.49)c	100.0
Bc7	49.8(1.70)a	33.3(1.53)a	54.3(1.74)a	38.0(1.58)ab	104.2(2.02)a	71.3(1.85)ab	100.0
Bc8	49.5(1.70)a	35.5(1.56)	56.3(1.75)a	39.8(1.61)a	103.3(2.02)a	75.3(1.88)a	100.0
Contr	0.0(0.00)c	0.0(0.00)c	0.0(0.00)c	0.0(0.00)d	0.0(0.00)c	0.0(0.00)d	0.0
Mean	33.8(1.41)	23.6(1.26)	39.6(1.46)	26.7(1.31)	75(1.69)	50(1.55)	
CV (%)	4.9	5.5	4.2	4.9	3.8	4.3	
Sign.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Mean not followed by the same letter differ significantly from one another according to Duncan multiple range tests, L-Lesion, CV–coefficient of variation, DAI–days after inoculation, AUDPC–Area under the progress curve, values in parenthesis are logarithmic transformation [log (x+1)], Sign. = significance level

4.4.2. Pathogenicity Test on Intact Faba Bean under Glasshouse Conditions

There was a highly significant ($P \leq 0.0001$) difference among the isolates of *B. cinerea* in disease severity and AUDPC. For this study, three virulent isolates (Bc1, Bc7 and Bc8) from detached leaf assay and five isolates (Bc10, Bc11, Bc13 and Bc17), which were not used in previous studies were screened. All the fungal isolates have infected the host even though their virulence levels were different (Figure 4.15, 4.16 and 4.17).

Similar to detached leaf assay, Bc7 and Bc8 showed the highest disease severity of 62.9 and 67.9%, respectively. In addition, the maximum progress of the disease symptoms over time (717.2 and 695.8%–days, respectively) were recorded on these isolates. However, control treatment showed the lowest disease severity (5.6%) and AUDPC (77.8%–days). Abdel Wahab (2015) revealed that there was a cellular degeneration in the hypo-virulent isolate as compared with the virulent isolate of *B. cinerea*. This study in agreement with the one conducted by Kwon and Park (2003) that the *B. cinerea* occurred severely on faba bean grew in a farmer`s field by showing discolored to gray or darker from the tip and eventually died.

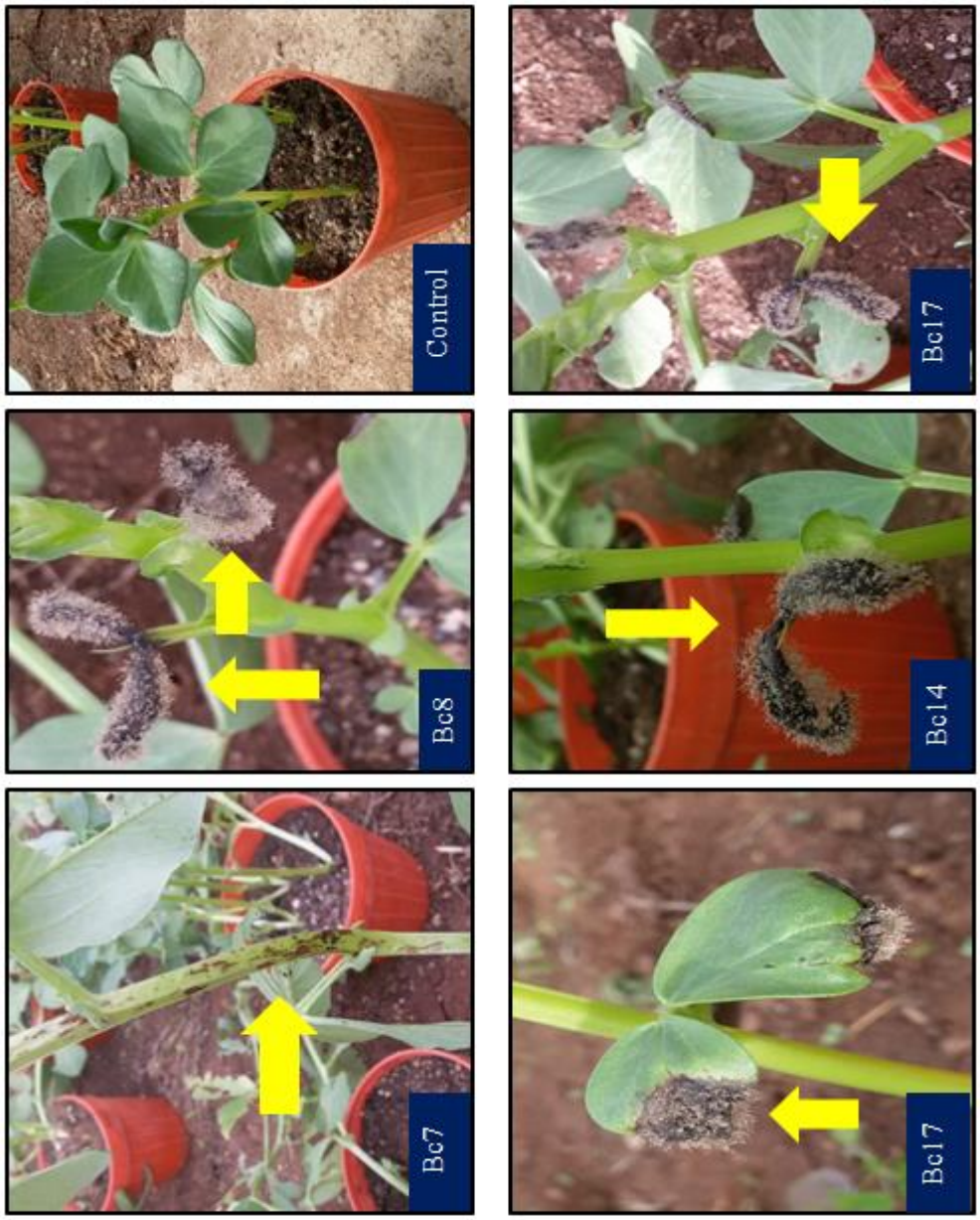

Figure 4.15. Isolates of *B. cinerea* showing high virulence in the pathogenicity test study under glass-house conditions in 2017

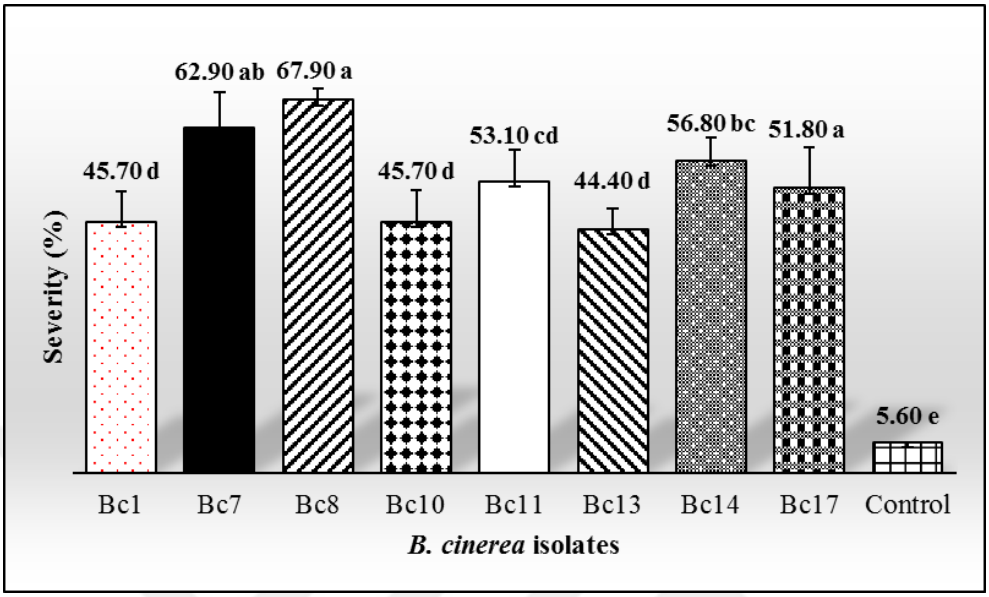

Figure 4.16. Disease severity of chocolate spot caused by isolates of *B. cinerea* under glass-house conditions, error bars=mean ± Standard deviation, Mean not followed by the same letter differ significantly from one another according to Duncan multiple range tests at 5%

A study conducted by Zhang et al. (2010) on three *Botrytis* species (*B. cinerea*, *B. fabae and B. fabiopsis*) causing faba bean chocolate spot revealed that pathogenicity test using the conidial suspension showed symptoms 7 days after inoculation. They revealed also disease severity index and the incidence of *B. cinerea* was lower than *B. fabiopsis* and *B. fabae.* Apart from this, the size of the leaf lesion produced by *B. cinerea* was less than the lesion size produced by *B. fabiopsis* and *B. fabae*. Gorfu (1996) demonstrated that there was virulence variation among *B. fabae* isolates, causing faba bean chocolate spot in Ethiopia. Isolates differed in their cultural characteristics, sclerotial production, and infection of different genotypes while they were similar in conidial size. Less virulent isolates of *B. fabae* formed smaller sclerotia than more virulent isolates and formed higher conidia

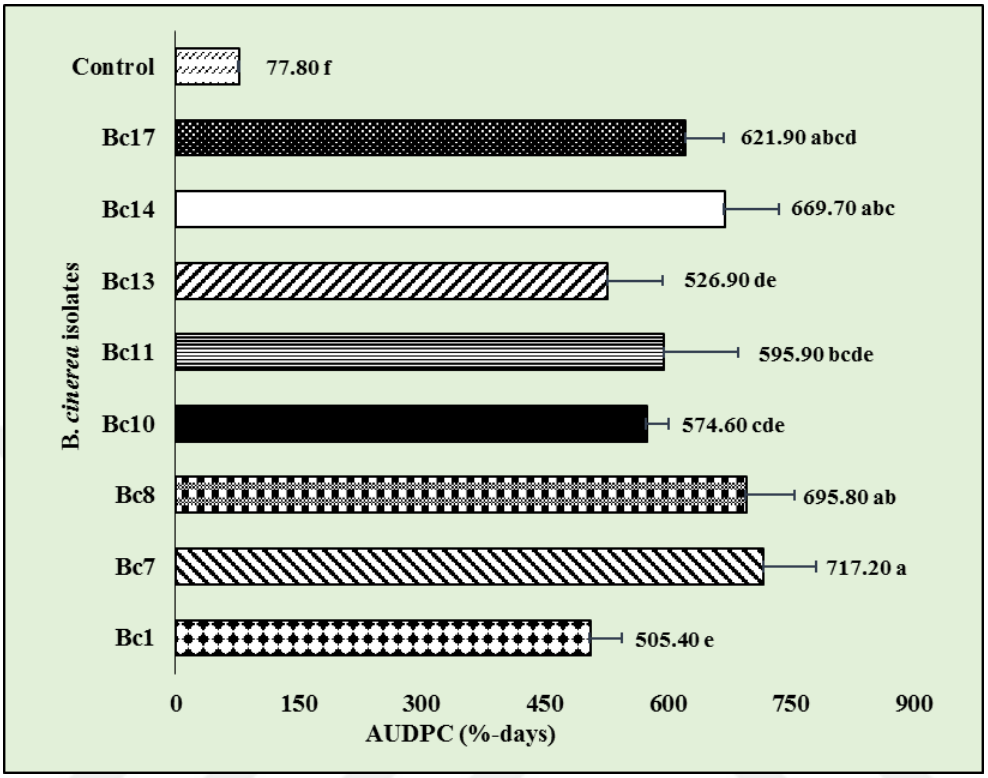

Figure 4.17. The area under disease progress curve of the chocolate spot caused by isolates of *B. cinerea* under glass-house conditions, error bars=mean ± Standard deviation, Mean not followed by the same letter differ significantly from one another according to Duncan multiple range tests

4.5. Biocontrol Efficacy of *T. harzianum* over a *B. cinerea* isolates

4.5.1. Antagonistic Assay by Dual Culture Method

The percentage of inhibition of radial growth was assessed for over 12 days. Because the Petri plates were incubated at 23 °C, both fungi grew normally. The percentage inhibition of radial growth (PIRG) values ranged from 70.0 to 84.0% for the 1st method and 46.0 to 59.0% for the 2nd method. The highest percentage inhibition of radial growths (84.0 and 59.0%) was scored with the interaction of Bc8 × T2 and Bc7 × T2 for the 1st method and 2nd method, respectively. Moreover, the highest inhibition zones (3.0 and 3.3 mm) were

recorded in Bc4 × T1 and Bc4 × T4 for the 1st method and 2nd method, respectively. Number of days for *T. harzianum* isolates to grow over *B. cinerea* isolates ranged from 7 to 13 and 10 to 14 days for the method I and method II, respectively (Table 4.8 and Figure 4.18).

In the current study, all tested *T. harzianum* isolates reduced the radial growth rate of *B. cinerea* both in the 1st and 2nd method, even though variations among isolates were observed in terms of degrees of inhibition. Similar to this study, Rahman et al. (2009) reported that *Trichoderma* strains can antagonize fungi growth to several capacities; their isolates within the same species may show different degrees of inhibition. Therefore, this study demonstrated that there was variation in the percentage inhibition of radial growth, inhibition zones and colony overgrowth day.

Table 4.8. Antifungal activity of *T. harzianum* isolates on the inhibition of radial growth, inhibition zone and colony overgrowth time of *B. cinerea* by dual culture technique

Treatment		At the periphery of the Petri dish (Method 1) (mean ± S.d)			20 mm away from the Petri dish periphery (Method 2) (mean ± S.d)		
B. cinerea	***T. harz***	**IRG%**	**The width of the zone (mm)**	**colony overgrowth time (day)**	**IRG%**	**The width of the zone (mm)**	**colony overgrowth time (day)**
Bc1	T1	77.8±4.6	4.17±1.0	13	55.7±1.3	2.67±0.3	12
	T2	74.7±4.6	3.33±0.3	14	59.0±2.2	2.33±0.6	10
	T3	79.8±4.6	4.33±0.3	12	55.7±3.8	2.83 ±1.0	11
	T4	73.7±4.6	3.33±1.5	15	55.1±5.1	2.67±0.6	13
	Mean	76.5±4.6	3.79±0.8	13.5	56.3±3.1	2.6±0.6	11.5
Bc4	T1	73.7±4.6	3.00±1.0	15	50.3±1.3	2.67±0.3	13
	T2	75.4±3.0	2.33±1.43	13	47.3±5.8	2.33±0.6	13
	T3	70.0±9.0	2.83±1.3	15	50.1±6.5	2.17±0.3	12
	T4	78.9±9.1	2.17±0.8	12	46.0±4.1	3.33±0.6	13
	Mean	74.5±6.4	2.6 ±1.1	13.8	48.5±4.4	2.6 ±0.5	12.8
Bc5	T1	79.5±0.8	4.00±0.0	12	55.6±5.2	2.67±2.3	12
	T2	84.0±10.3	4.67±0.6	12	56.9±2.8	2.50±2.2	11
	T3	77.9±2.6	4.00±0.0	13	56.2±3.6	2.67±0.3	11
	T4	76.0±7.0	4.83±0.3	13	55.0±4.8	2.83±1.3	12
	Mean	76.6 ±5.2	3.5 ±0.2	12.5	53.4 ±4.1	2.6 ±1.5	11.5
Bc7	T1	81.8±2.2	2.17±0.8	12	52.1±2.6	1.33±1.2	13
	T2	84.0±10.3	1.50±1.5	13	49.8±4.8	1.67±1.5	14
	T3	76.6±3.9	2.67±1.2	13	51.9±7.3	2.33±0.6	13
	T4	80.5±3.9	1.67±0.3	13	49.9±3.5	1.33±1.2	14
	Mean	80.8±5.2	2.0±1.0	12.75	50.9±4.6	1.7±1.1	13.5
Bc8	T1	75.8±3.8	1.50±0.0	14	50.5±7.6	1.00±0.9	13
	T2	74.7±10.1	2.50±0.5	15	52.9±4.2	2.67±0.3	12
	T3	70.3±3.3	1.17±1.0	15	50.1±6.2	1.67±0.3	13
	T4	72.5±5.0	3.33±0.6	14	52.8±4.8	2.33±0.3	12
	Mean	73.3±5.6	2.1±0.5	14.5	51.6±5.7	1.9±0.5	12.5

IRG: inhibition of radial growth, S.D=standard deviation

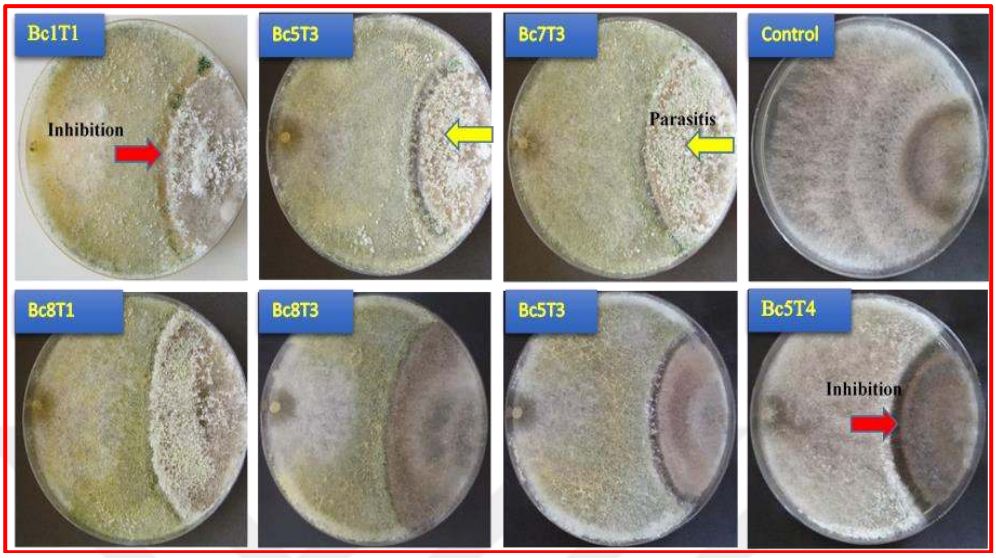

Figure 4.18. Effect of *T. harzianum* on mycelial growth of *B. cinerea* isolates

4.5.2. Antagonism Test on Detached Leaves

For this experiment, 10 *T. harzianum* (T1, T2, T3, T4, T7, T8, T9 and T10) were used. The analysis of variance (ANOVA) of disease severity for 5 and 7 days after inoculation and the area under the disease progress curve (AUDPC) showed highly significant ($P \leq 0.001$) difference among the treatments. As described in Table 4.9, all isolates of *T. harzianum* reduced disease epidemics on detached faba bean leaves. It was clearly noticed that first black gray spots were observed 72 hours after inoculation of a pathogenic fungus, and the symptom increased with time. However, on control leaves, the first symptom was observed 48 hours after the fungus inoculation. The lowest disease severity (37.5%) and area under disease progress curve (65.6%-days) were calculated on T8 and T9 on the final day of disease assessment whereas leaves treated with *B. cinerea* alone exhibited the highest disease severity (68.8%) and AUDPC (131.3%-days).

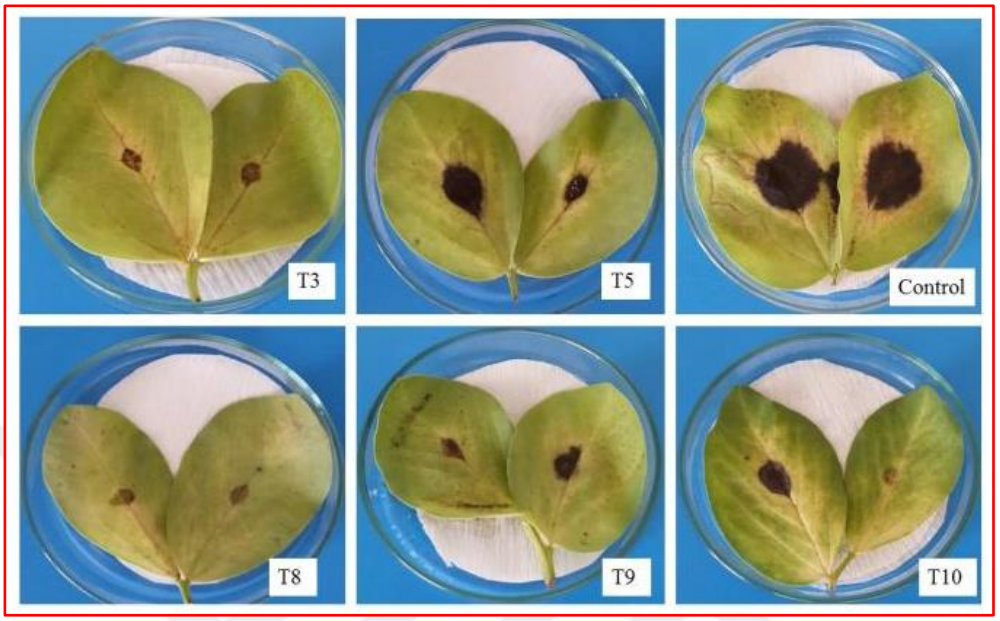

Figure 4.19. Antagonistic evaluation of different *T. harzianum* against the chocolate spot on detached leaves

In this study, isolates of *T. harzianum* showed variability in reducing the severity developed by *B. cinerea* on detached faba bean leaves. Nevertheless, all *Trichoderma* isolates could not prevent the number of faba bean (incidence) infected with the fungus, which showed a 100% disease incidence (Figure 4.18). Tivoli et al. (2006) reported that a detached leaf test is a rapid and simple *in vitro* test, which aimed to confirm the results obtained from *B. fabae* screening test in the *in vivo* conditions. In this study, the antagonistic assay against *B. cinerea* was conducted at 23±2 °C because this pathogen grows best at the temperature range of 20 to 25 °C, this might be due to the fact that growth and antagonistic activities of *T. harzianum* inhibited with either decrease or increase in temperature. As reported by Grigoriev et al. (2012), *T. atroviride* is well known for its biological control against different phytopathogenic fungi like *Botrytis cinerea* and *Rhizoctonia solani* that are major pests of crops, including beans, cotton, grapes, tomatoes, strawberries and cucumber.

Table 4.9. Antagonistic evaluation of ten isolates of *T. harzianum* against disease epidemics of *B. cinerea* on detached faba bean leaves

Treatment	Mean ±SE			
	Severity 5 DAI	Severity 7 DAI	AUDPC (%-days)	Incidence %
T1	34.4±3.1bc	43.8±3.6bc	78.1±5.9bcd	100
T2	37.5±0.0bc	46.9±3.1bc	84.4±3.1bc	100
T3	43.8±3.6b	50.0±0.0b	93.8±3.5b	100
T4	34.4±3.1bc	43.7±6.2bc	78.1±7.9bcd	100
T5	37.5±5.1bc	46.8±5.8bc	84.4±10.6bc	100
T6	28.1±3.1c	43.7±3.6bc	71.8±5.8cd	100
T7	34.3±3.2bc	43.8±3.5bc	78.1±3.1bcd	100
T8	28.1±3.1c	37.5±0.0c	65.6±3.2d	100
T9	28.1±3.1c	37.5±0.0c	65.6±3.1d	100
T10	37.5±0.0bc	43.7±3.5bc	81.3±3.6bcd	100
Control	62.5±0.0a	68.8±3.6a	131.3±3.6a	100
Mean	36.9±1.6	46.0±1.6	82.9±2.9	100
CV (%)	16.1	16	13.1	
Sign.	<0.0001	0.0002	<0.0001	

DAI: days after inoculation, AUDPC: Area under disease progress curve, numbers with similar letter do not differ significantly at the 5% level according to Duncan's multiple range test (DMRT), Sign. = significance level, SE=standard error

4.5.3. Antagonism Test under Glasshouse Conditions

There was a significant ($P \leq 0.001$) difference among the treatments in reducing both disease severity under glasshouse conditions (Figure 4.20 and 4.21) in both years of 2016 and 2017. Disease severity was scored three times for both experiments conducted in 2016 and 2017 starting from disease onset, which was 7 days after pathogen (*B. cinerea*) inoculation.

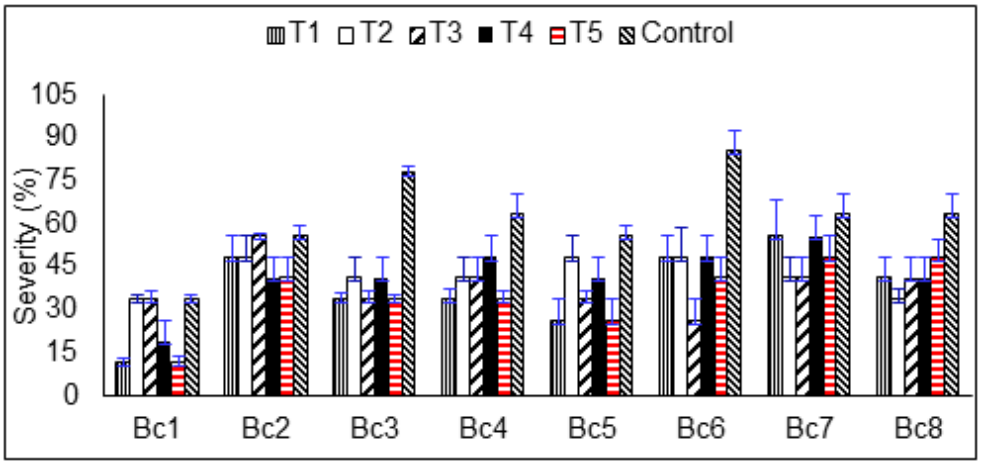

Figure 4.20. Effects of different isolates of *T. harzianum* on chocolate spot severity caused by *B. cinerea* under glasshouse conditions (CV=25.2%, Sign.=0.0001, error bars=mean ± Standard deviation) in 2016

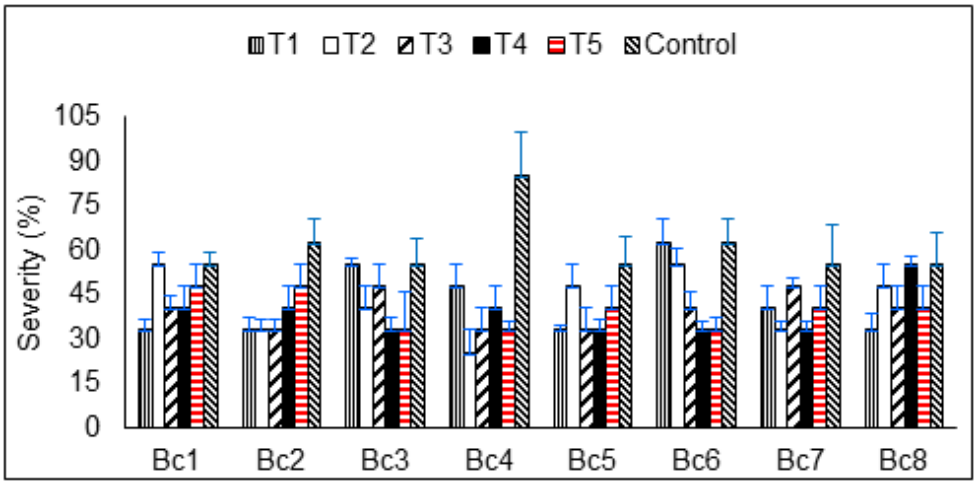

Figure 4.21. Effects of different isolates of *T. harzianum* on chocolate severity caused by *B. cinerea* under glasshouse conditions (CV=22.7%, Sign. =0.0001, error bars=mean ± Standard deviation) in 2017

The highest disease severity was scored on control pots, which inoculated with *B. cinerea* alone in both years. While the lowest was scored on the pots inoculated with the interaction of *T. harzianum* and *B. cinerea* isolates, even though the antagonistic ability of this fungal bio-agent and virulence of the

pathogenic fungus were different. The interaction of each Bc1 × T1 and Bc1 × T5 reduced disease severity by 66.7%, while Bc7 × T4 reduced the disease by 11.76 % in 2016. In addition, the area under the disease progress curve (AUDPC) showed a significant difference (($P \leq 0.001$) between the treatments (Figure 4.22 and 4.23) in both years. The maximum AUDPC (1037.0 and 751.9 %-days) were calculated on control (Bc3 × T0 and Bc4 × T0) pots in the first and second experiment, respectively. While the minimum (155.6%-days) was scored on a Bc1 × T1 pot of the first experiment. In addition, all posts were infected with a chocolate spot showing 100% disease incidence in both first and second experiments. Therefore, *T. harzianum* isolates, identified on faba bean and field pea showed the promising result in controlling faba bean chocolate spot.

Results from all tested isolates of *T. harzianum* under glasshouse conditions were able to reduce chocolate spot disease epidemic such as disease severity and area under disease progress curve; however, the antagonistic ability of the isolates varied. Isolates numbered T1 and T5 reduced the severity of the disease more than other isolates in 2016.

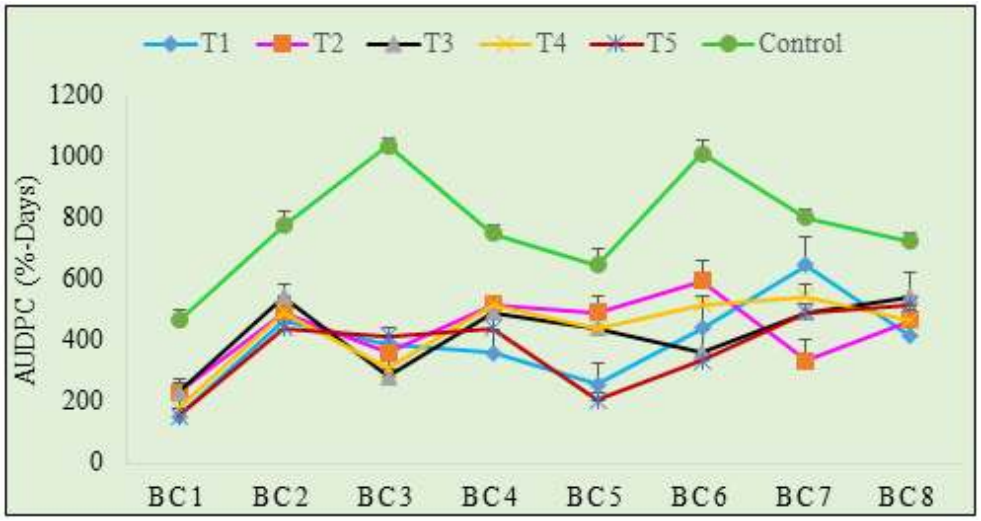

Figure 4.22. Effects of different *T. harzianum* on AUDPC caused by *B. cinerea* under glasshouse conditions (CV=17.3%, Sign.=0.0001, error bars=mean ± Standard deviation) in 2016

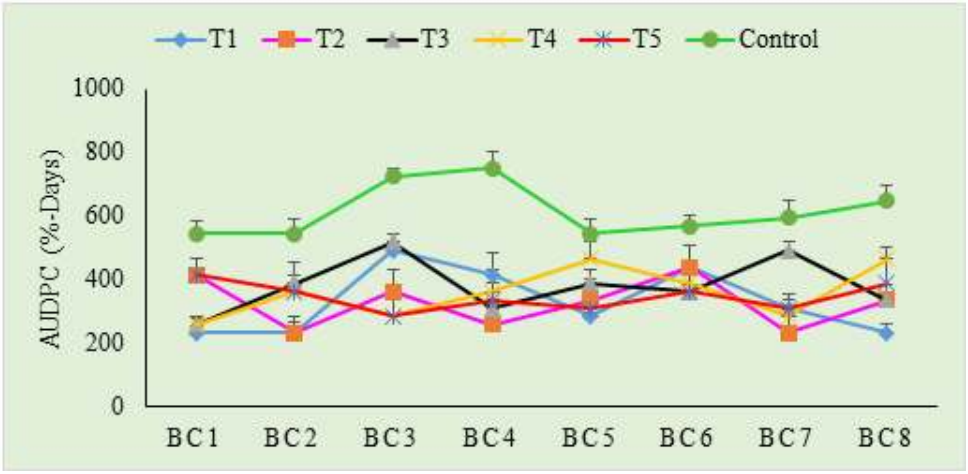

Figure 4.23. Effects of different *T. harzianum* on AUDPC caused by *B. cinerea* under glasshouse conditions (CV=20.8%, Sign. =0.0001, error bars=mean ± Standard deviation) in 2017

4.5.4. Evaluation of Selected *T. harzianum* Isolates under Field Conditions

Effect of *T. harzianum* isolates was tested against chocolate spot development on faba bean seedlings during 2017 and 2018 seasons under field

conditions. Application of *T. harzianum* as a foliar spray significantly (P≤0.0001) reduced the extent of chocolate spot disease epidemics (severity and area under disease progress curve) on faba bean seedlings as compared with control plots, which were inoculated with *B. cinerea* alone in both years, even though the epidemic degree in 2018 was higher than that of 2017. This was because of the weather conditions of 2018 was more conducive for pathogen than antagonistic fungus (Figure 4.20, 4.21 and 4.22).

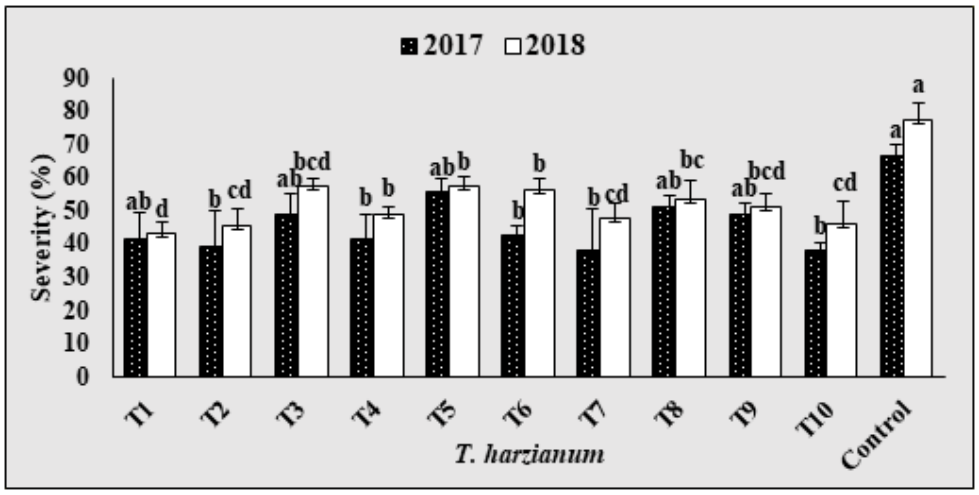

Figure 4.24. Effect of *T. harzianum* isolates on disease severity of faba bean chocolate spot under field conditions during 2017 and 2018 seasons, Mean not followed by the same letter differ significantly from one another according to Duncan multiple range tests

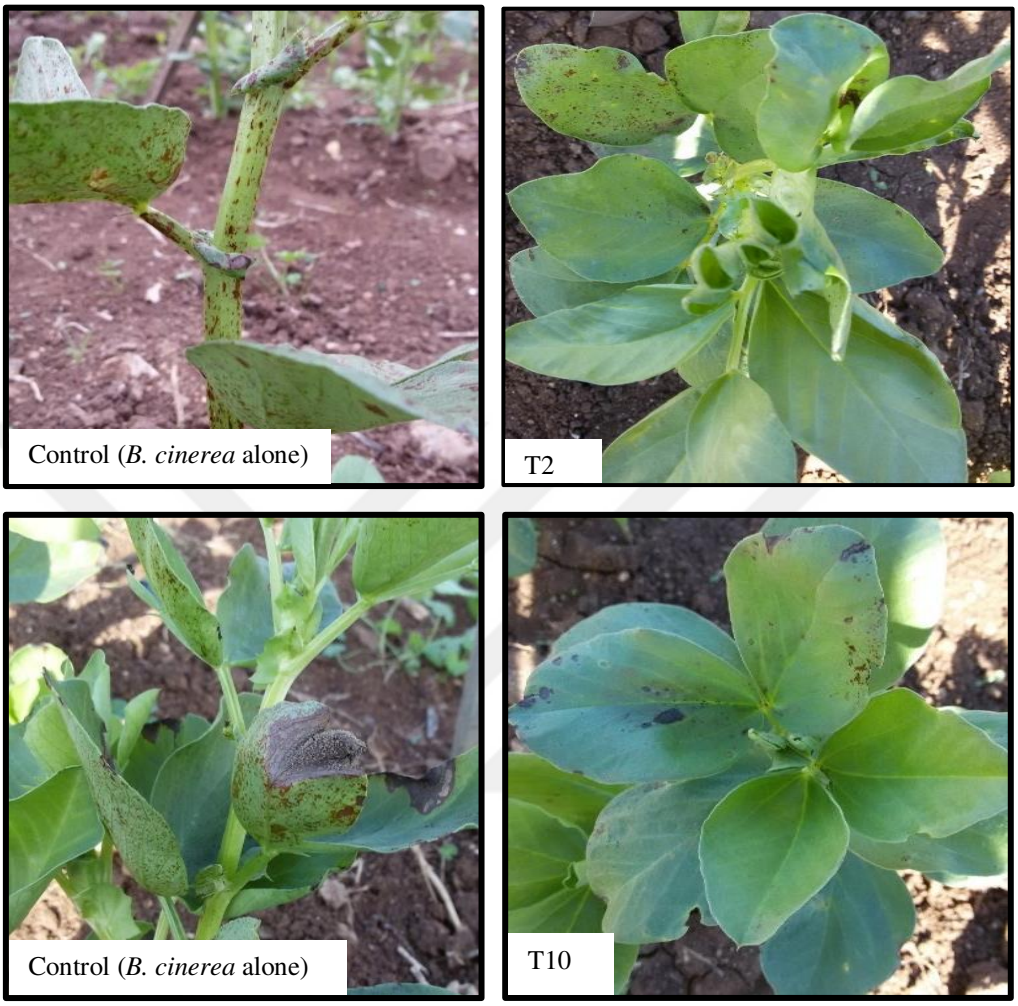

Figure 4.25. Effect of *T. harzianum* on faba bean chocolate spot disease epidemics under field conditions

This study exhibited that the effect of *T. harzianum* isolates on the faba bean chocolate spot caused by *B. cinerea* showed the promising result. Accordingly, T2, T7, and T10 reduced the disease severity by 41.6, 43.2 and 43.1% respectively in 2017 experiment and the lowest AUDPC (396.5%-days) was scored on faba bean seedlings sprayed with *T. harzianum* isolate (T6) while the maximum was calculated on the control plot (707.8%-days) (Figure 4.21). Moreover, T1, T2 and T10 reduced the chocolate spot disease severity on faba

bean by 44.1, 41.4 and 40.4%, respectively, in the 2018 experiment. Furthermore, the maximum AUDPC (959.2%-days) was calculated on control plots, while the minimum (508.1 and 534%-days) calculated on T1 and T5, respectively (Figure 4.22). A study conducted by El-kholy (2014) showed that *B. faba* is more sensitive to fungicides than *B. cinerea* under field conditions. Therefore, alternative control measures against *B. cinerea* fungus, including *T. harzianum* should get attention in the future.

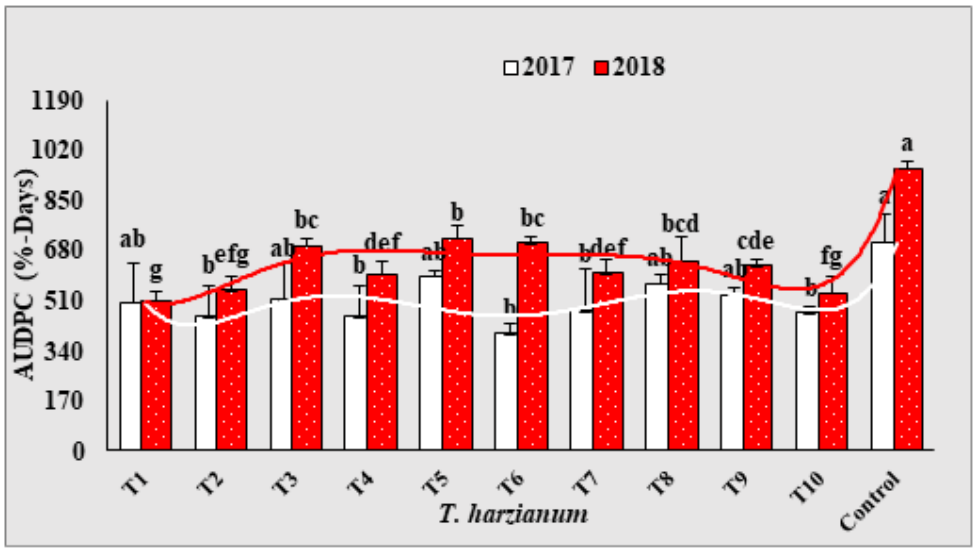

Figure 4.26. Effect of *T. harzianum* isolates on AUDPC of faba bean chocolate spot under field conditions during 2017 and 2018 seasons, Mean not followed by the same letter differ significantly from one another according to Duncan multiple range tests

5. CONCLUSIONS AND RECOMMENDATIONS

Faba bean (*Vicia faba* L.) occupies 4th rank among legume crops in terms of production, after chickpea, lentil and dry bean in Turkey. However, the crop is constrained by chocolate spot disease caused by *B. cinerea* in Adana province of Turkey mainly during pods setting to ripening. In Ethiopia, the crop is mainly infected and devastated by *B. fabae*. In a present study, cultural and morphological features, namely a growth pattern; sclerotia and conidia as well as conidiophore characteristics; molecular identification the fungus using ITS and Beta-tubulin, and the effect of different temperatures on the growth of the fungus, were investigated. However, ITS primer is not an ideal candidate for *Botrytis* species identification because all isolates' sequences showed homogenous. Moreover, *B. fabae* isolates were brought from Ethiopia and included in PCR study. The optimum temperature ranges for the growths *B. cinerea* and *T. harzianum* were 15-20 °C and 25-30 °C, respectively.

Eight isolates of *B. cinerea* were subjected to the pathogenicity test, and all isolates were able to cause disease, however, isolates collected in 2015/2016 from faba bean stems, Bc7 and Bc8, were more virulent than the others both on detached faba bean leaves and intact plant. These isolates were used for antagonism tests both under *in vitro* and *in vivo* conditions. Apart from these, isolates of *T. harzianum* were successfully isolated from faba bean and field pea of leaves and stems, identified using molecular method (ITS and tef1-α) and morphological characterization. Finally, their antagonistic evaluation against *B. cinerea* isolates was carried out both under *in vitro* and *in vivo* conditions. As a result, *T. harzianum* isolates, T1, T7 and T10, showed promising results both under glasshouse and field conditions. Based on these results, the following recommendations were listed:

1. Current study confirmed that under the conducive environmental condition, *B. cinerea* pathogen can cause a serious problem during the winter period. Therefore, attention should be given also to *B. cinerea* in addition to *B. fabae* and *B. fabiopsis*. Future works should have to address yield losses caused by *B. cinerea* on faba bean mainly in current study areas.
2. In a country like Turkey, where faba beans are consumed as a vegetable, controlling *B. cinerea* using fungicides may not be advisable, It may also develop resistance against fungicides which are unsafe to the human health. Therefore, the use of alternative disease management strategies like use of *Trichoderma* spp. need to be explored.
3. From the antagonistic assay results, it was noticed that some *T. harzianum* isolates were more effective than the others in reducing disease epidemics (severity and AUDPC). Therefore, it is advisable to screen the efficacy of bio-agents of the same strains or different species under field conditions before use.
4. Up to date, approximately more than 90% of *Trichoderma* species have been isolated and characterized from plants' and or crops' rhizosphere. However, the current study pointed out that it is possible to isolate *Trichoderma harzianum* easily from faba beans' and field peas' phyllosphere. Therefore, future studies should pay attention to several substrates, mainly, legume crops, forest plants, where several bio-diversities may available. They should also be tested against this fungus.
5. *Trichoderma* spp. including *T. harzianum* have to be part of integrated disease management, like combining with appropriate intra and inter-row spacing, the use of resistant cultivars, adjusting the time of sowing, and even with other biocontrol agents.

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