CUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Waleed Khalid Ahmed AHMED

ISOLATION AND MOLECULAR DIAGNOSIS OF TRICHODERMA SPP. AND EVALUATION OF IT'S INHIBITORY EFFECTIVENESS ON SOME PATHOGENIC FUNGI

DEPARTMENT OF BIOTECHNOLOGY

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We certify that the thesis titled above was reviewed and approved for the award of degree of the Doctor of Philosophy by the board of jury on 20/05 /2020.

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ABSTRACT

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In this study.**4** isolates of *Trichoderma spp*. as well as 7 isolates of *Macrophomina .phaseolina* and one isolate of *Fusarium* were isolated and identified from soil and compost in Adana province / Turkey. In addition Two isolates *Dermatophytes* sp, were included for molecular study.The identification of *Trichoderma spp* was carried out based on morphological features as well as phylogenetic analysis using two primers (ITS1 and ITS4). Isolates were identified as T. *viride, T. virens,T. harzianum* and *T. lixii*. while all isolates of *M.phaseolina* were identified as *M.phaseolina* based on morphological features as well as phylogenetic analysis using two primers (ITS5 and ITS4). Also isolates of *Fusarium* and *dermatophytes* were identified as *F.oxysporum, Trichophyton tonsurans* and *Microsporium canis* respectively based on phylogenetic analysis using two primers (ITS1 and ITS4).

From the antagonistic test. all isolates of *Trichoderma* showed promising results in reducing growth of pathogens under *in vitro* conditions. However, the efficacy was variable among the tested isolates.The best antifungal concentration was (15 ml/L) with *T. harzianum* isolate in inhibition for *M.phaseolina, F.oxysporum*, *Trichophyton tonsurans* and *Microsporium canis.*

Keywords: *Trichderma spp*, Molecular diagnosis,*M.phaseolina*, *dermatophytes*

ÖZ

DOKTORA TEZİ

TRICHODERMA SPP. IZOLASYONU VE MOLEKÜLER TANISI VE BAZI PATOJENIK FUNGUSLAR ÜZERINDEKI ENGELLEYICI ETKINLIĞININ DEĞERLENDIRILMESI.

Waleed Khalid Ahmed AHMED

ÇUKUROVA ÜNİVERSİTESİ
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Bu çalışmada Adana bölgesinde toprak ve komposttan dört *Trichoderma* spp. yedi M.phaseolina ve bir Fusarium izolatı tanımlandı. Buna ek olarak moleküler çalışma için iki dermatofit izolatı da çalışmaya dahil edildi. *Trichoderma* spp'nin filogenetik analizi morfolojik özelliklerine dayanarak ve iki primer (ITS1 ve ITS4) kullanılarak yapıldı. A; *T. viride*, S; *T. virens*, H; *T. harzianum* ve ASH; *T. lixii* olarak tanımlandı. M.phaseolina'nın tüm izolatları *M.phaseolina* olarak tanımlandı. Ayrıca *Fusarium* ve dermatofit izolatları, morfolojik özelliklere ve iki primer (ITS1 ve ITS4) kullanılarak filogenetik analizlere dayanarak sırasıyla *F.oxysporum*, *Trichophyton tonsurans* ve *Microsporium canis* olarak tanımlanmıştır.

Antigonistliğin test edildiği deneyde,*Trichoderma* izolatının tüm izolatları in vitro şartlarda patojenlerin (*M.phaseolina, F.oxysporum, Trichophyton tonsurans* ve *Microsporium canis*.) büyümesini azaltmada umut verici sonuçlar göstermiştir, ve en aktif antifungal konsantrasyonu 15ml/L idi, ile *T. harzianum* izolatı.

Anahtar kelimeler: *Trichderma spp*, Moleküler tanı ,*M.phaseolina*,*dermatophytes*

EXPANDED ABSTRACT

M. phaseolina is an important soilborne pathogen. It can infect of more than 700 plant species . Besides being an opportunistic plant pathogen, several clinical reports have also established *M. phaseolina* as an intermittent human pathogen that may cause cutaneous and several fungal infections, especially a in immune-suppressed patients undergoing prophylactic antifungal treatments. *F. oxysporum* also is a double disease for plants and humans.

In this study was determine the identify of *Trichoderma* isolates and pathogens isolates using molecular method, Molecular identification of the *Trichoderma spp*. based on combined DNA sequence data as well as morphological characteristics the results showed that isolates collected from the soil and compost were identified as *T. viride, T. virens, T .harzianum* and *T. lixii.* while the pathogens were identified as *M.phaseolina*, *F.oxysporum,T.tonsurans* and *M. canis*.and finally to evaluate the efficacy of isolates of *Trichoderma* against *M. phaseolina, F. oxysporum, T.tonsurans* and *M. canis* under *in vitro* conditions in both methods concentration from filtres of *Trichoderma* isolates and dual culture technique. Based on the results obtained, isolates of *Trichoderma* showed inhibition of plant and human pathogens.

Antagonistic assessment of different isolates of *Trichoderma* against pathogens (*M.phaseolina, F.oxysporum,T.tonsurans* and *M canis*) by different concentrations assay (5,10and 15 ml/L) showed variation in percentage of inhibition zone. The inhibition zone percentage growth values ranging in all concentrations and isolates from 37.06 to 100.% for a *F.oxysporum*. while the percentage inhibition of *M.phaseolina* ranging from 40.80 to 100% . Moreover,the highest percentage inhibition of zone growth (100 and100%) was scored with *T. harzianum* isolate in 15ml/L concentration,for both pathogens.

The results of antagonistic assay against *T.tonsurans* and *M .canis* revealed that the highest percentage inhibition was scored, which was treatment

with *T. harzianum* in 15ml/L concentration, in which reached 93.75 and 94.17 % respectively.

For antagonistic assessment of different isolates of *Trichoderm* against plant and human pathogens (*M.phaseolina, F.oxysporum,T.tonsurans* and *M canis*) by dual culture assay showed variation in inhibition zone . Moreover, the highest inhibition zones (1.33 and 1.66 cm) were recorded with *T. harzianum* against *F.oxysporum* and *M.phaseolina*, respectively.

Antagonistic test against *T.tonsurans* and *M canis* showed that all isolates of *Trichoderma* reduced growth of pathogens. However, The highst values inhibition with *T. harzianum* isolate in which reached 1.34 and 1.50 cm, respectively.

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LIST OF SYMBOLS AND ABBREVIATONS

%	: Percentage
°C	: Degree Celcius
bp	: base pair
DNA	: Deoxyribonucleic Acid
µl	: Microlitres
PCR	: Polymerase Chain Reaction
TE	: Tris/EDTA
ITS	: Internal transcribed spacer
CTAB	: Cetyltrimethyl ammonium bromide
CRD	: Completely randomized design
Cm	: Centimeter
spp	: Species
NCBI	: National Center for Biotechnology Information
PDA	: Potato Dextrose Agar
PDB	: Potato Dextrose Broth
SDA	: Sabouraud's Dextrose Agar
T	: *Trichoderma*
M	: *Macrophomina*
F	: *Fusarium*
M	: *Microsporum*
T	: *Trichophyton*
LSD	: Least significant difference
ml	: milliliter
L	: Liter
SAS	: Statistical analysis system
M.p	: *Macrophomina phaseolina*

1. INTRODUCTION

Rare and emerging opportunistic fungal pathogens are a serious and growing concern for immunosuppressed patients. ***Macrophomina phaseolina***, an important soilborne plant pathogen in tropical and subtropical soil, should be considered in this category. It may be evident as a localized cutaneous cellulitis or as an ocular keratitis (Schwartz & Kapila, 2019). considerable several clinical reports have also classified M. phaseolina as an infrequent pathogen, which could infect humans and cause, afterwards, various fungal or cutaneous infections (Srinivasan et al., 2009; D. H. Tan, Sigler, Gibas, & Fong, 2008), It's also consiedered one of the Soilborne pathogens cause significant economic losses in crop production. They affect crops from their initial stages in nurseries until harvest. Plant diseases caused by *Macrophomina phaseolina Rhizoctonia spp., Fusarium spp., Verticillium spp., Sclerotinia spp., Pythium spp.,* and *Phytophthora spp*. affect a number of important crops, including wheat, cotton, vegetables and temperate fruits. ***M. phaseolina*** is an important soil and seed-borne pathogen, Its can infecte of more than then 700 of plant species (Csöndes, Cseh, Taller, & Poczai, 2012; Ijaz, Sadaqat, & Khan, 2013; Ndiaye, Termorshuizen, & van Bruggen, 2010). Economic losses of *M.phaseolina* are estimated at 60-90% for many crops (Arias, Ray, Mengistu, & Scheffler, 2011).

Charcoal rot is of prime importance in reducing crop yield especially in arid regions of the world. It is an important disease during hot and dry weather or when unfavorable environmental conditions prevails (O. D. Dhingra & Sinclair, 1978). Tropical crop plants are seriously affected by this pathogen (Malaguti, 1990). *M. phaseolina* is classified as a Deuteromycete which shows two asexual sub-phases, a mycelial phase named as *Rhizoctonia bataticola* (Taub) and the other a pycnidial phase called *M. phaseolina* (O. D. Dhingra & Sinclair, 1978). The fungus is classified in the Botryosphaeriaceae within Ascomycota phylum according to recent phylogenetic data (Crous et al., 2006). The fungus produces

dark brown lesions on the epicotyls and hypocotyls of seedlings. The seedling dies because of obstruction of xylem vessels. In adult plants, the pathogen causes red to brown lesions on roots and stems and produces dark mycelium and black microsclerotia. The stem shows longitudinal dark lesions and the plant becomes defoliated and wilted (Abawi, 1990). The black, 0.1-1 mm sized microsclerotia are formed in soil, infected seeds or host tissues and constitute the primary inoculum source of the pathogen (D Bouhot, 1968). They can survive up to 15 years depending on environmental conditions, and whether or not the sclerotia are associated with host residues (G. Cook, Boosalis, Dunkle, & Odvody, 1973; G. Short, Wyllie, & Bristow, 1980). *M. phaseolina* is a heat tolerant pathogen since sclerotia could withstand a temperature range of 60-65C (Bega & Smith, 1962; J. Mihail & Alcorn, 1984) . The evidence suggests that it is primarily a root inhabiting fungus and produces tuber or cushion shaped 1-8 mm diameter black sclerotia. These sclerotia serve as a primary means of survival (Kaiser & Horner, 1980; Smith, 1969).

Fusarium oxysporum is a species complex that encompasses genetically and phenotypically diverse strains and has been found in a wide range of ecosystems. Most studies on the *F. oxysporum* species complex have focused on plant pathogenic strains because they cause diseases in 120 agriculturally and horticulturally important plants (Michielse & Rep, 2009). In a recent survey in the international community of fungal pathologists, *F.oxysporum* was ranked fifth in a list of top 10 fungal plant pathogens based on scientific and economic importance (Dean et al., 2012). Besides infecting diverse plants, certain members of the F. oxysporum also cause localized or deeply invasive infections in humans, causing very high mortality in immunocompromised patients (O'Donnell et al., 2009; Ortoneda et al., 2004). Fo can also infect the corneas of immune-competent humans, frequently causing blindness, and was associated with outbreaks of Fusarium keratitis in Asia and the United States (Chang et al., 2006; O'Donnell, Cigelnik, & Nirenberg, 2007).

Trichoderma species are cosmopolitan fungi, frequently present in all types of soil, manure (Alexander, 1961). The first description of a fungus under the genus *Trichoderma* dates back to 1794 (Persoon, 1794) and in 1865 a link to the sexual state, Hypocrea, was suggested by Tulasne and Tulasne (1865). This anamorph–teleomorph relationship was only confirmed more than 100 years later for *Trichoderma* reesei and *Hypocrea jecorina* (Kuhls et al., 1996). *Trichoderma* is a well-studied genus of fungi that currently comprises of more than 200 genetically defined species (Atanasova et al., 2013). *Trichoderma spp.* (Phylum Ascomycota, Order Hypocreales, Family Hypocreaceae) live in soils all over the world, in all climatic zone, and are characterised by their rapid growth and resistance to noxious substances. Usually they exercise a dominant role in the ecosystem of soil fungi (Danielson & Davey, 1973; Klein & Eveleigh, 1998; Roiger, Jeffers, & Caldwell, 1991; Widden, 1979; Widden & Abitbol, 1980) ,as they are important decomposers of woody and herbaceous materials (Rossman, 1996). Fast growth and the ability to employ a vast variety of nutrients make them also necrotrophic against many primary wood decomposers like white and brown rot fungi of the Basidiomycetes (De Marco, Valadares-Inglis, & Felix, 2004).

Actually, *Trichoderma spp*. reproduces asexually through conidia spore production on conidiophores that have branched structures and develop on hyphal tips. The conidia spores are green, yellow, or white, which is one of the characteristics of the fungi. *Trichoderma* species are opportunistic, avirulent plant symbionts with known potential to control plant diseases (Harman, Howell, Viterbo, Chet, & Lorito, 2004). There are other beneficial functions of *Trichoderma*, such as plant growth promotion and tolerance induction to abiotic/biotic stresses (Hanhong, 2011).

Some *Trichoderma* species such as *T. harzianum, T. virens*, and *T. viride* are commercially marketed as biopesticides (Grondona et al., 1997) . There are several mechanisms through which *Trichoderma spp*. control plant pathogens, including. Fungi belonging to the genus *Trichoderma* are soil borne, green spored ascomycetes that are found in nearly all ecological niches. They have various characteristics and applications that allow them to be successful colonisers of their habitats, efficiently fighting their competitors and establishing a predominant role in their environment (Felix et al., 2014). *Trichoderma* is considered as one of the efficient biocontrol agent due to its high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in nutrient utilization, capacity to modify the rhizosphere, strong aggregativeness against the pathogenic fungi and efficiency in promoting plant growth and defense mechanisms (Grondona et al., 1997).

It is known to control soil borne fungal pathogens belonging to class Ascomycete, deutaromycete, Bascidiomycete and also certain air borne pathogens (Al-Obaidy & Al-Rijabo, 2011; Chilosi et al., 2020; Monte, 2001; Ziedan, Moataza, & Eman, 2005), also Dermatophytes pathogens(Aasi & Al-Aaraji, 2018; Al-Rijabo & Al-Obaidy, 2011; Omero, Dror, & Freeman, 2004).

Therefore, soil contains a variety of disease causing by organisms for both plants, animals and human. These include bacteria, fungi, protozoa, Actinomyces, phytoplasmas, and viruses, which called soil borne pathogens. For several plant diseases, soil can be treated as its reservoir where the organisms residing in the soil until the appropriate conditions are strained. Indeed, soil conditions play a pivotal role in plant disease affecting the survival of the pathogen. Among theme, fungi are considered as the most important soil borne pathogens. These latest, are filamentous, multicellular, eukaryotic, and they are heterotrophic organisms that create chain of hyphae named the mycelium and uptake nutrients from the enclosing substrate (Alexopoulos et al. 1996).

In fact, the dermatophytoses are one of the most frequent human disease. Admitting, the prevalence of those infections varies greatly and at least 10–20% of the world population may be infected with dermatophytes (Hryncewicz-Gwóźdź, Jagielski, Dobrowolska, Szepietowski, & Baran, 2011). The dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair, nail, feathers, horns and hooves) of human and other animals to produce an infection, dermatophytosis. Well, around 40 species belonging to the genera *Microsporum, Trichophyton and Epidermophyton* are considered as dermatophytes They possess two important properties, keratinophilic and keratinolytic. This mean that they have the ability to digest keratin in vitro in their saprophytic state and utilise it as a substrate, and some may invade tissues in vivo (Kalinowska K, Hryncewicz-Gwóźdź A, & E., 2009). According to their host preference and natural habitat, dermatophytes are generally grouped in three categories, anthropophilic (human), zoophilic (animal) and geophilic (soil) (Youngchim, Pornsuwan, Nosanchuk, Dankai, & Vanittanakom, 2011).

Since soil borne and dermatophytes pathogens as well as symbionts, share common habitat and show differential influence of the host plant and human , major interest has recently focused on the relevance of *Trichoderma* species in the control of soil borne pathogen (Dar, Zargar, & Beigh, 1997; Srivastava, Singh, Jana, & Arora, 2001).

On view of this, in this study we selected certain *Trichoderma* species after isolate from soil and compost to control against plant and human pathogens in order to increase biological control against about the spread pathogens . Due to the importance of soil borne pathgens on crops and dermatophytes on human this study aim to use Trichoderma species in the control of this diseases in order to find a chemical- free resistance method. The study aim of this study is:

1.isolation and molecular diagnosis of ***Trichoderma spp***.

2.Evaluation of the effectiveness of ***Trichoderma spp*** in the control of plant pathogens like as ***M.phaseolina*** and ***F. oxysporum*** In addition to dermatophytes pathogens such as ***Trichophyton*** and ***Microsporum.***

2. LITERATURE REVIEW

2.1.*Macrophomina Phaseolina*

Charcoal rot disease caused by ***M. phaseolina*** is a common soil-borne fungus often known by its imperfect state Macrophomina phaseolina (Tassi) Goid. It was first reported in India by Uppal (1931 and 1934). It is the pycnidial stage of Rhizoctonia bataticola (Ashby 1927) with the following synonyms.

Botryodiplodia phaseoli (Maubl.)

Thirum Dothiorella cajani Syd,. P. Syd. and E. J. Butl.

Manophoma cajani Syd., P. Syd. adn E. J. Butl.

Macrophomina phaseoli Maubl.

Rhizoctonia lamellifera Small

Sclerotium bataticola Taubenh

Rhizoctonia bataticola (Taubenh) E. J. Butler (anamorph).

Different other synonyms was assigned to the fungus, including *Macrophomina phaseoli* (Maubl.) Ashby, Macrophoma conchoci Swada, *Sclerotium bataticola* Taub and *R. bataticola* (Taub). Because of unknown teleo morph its taxonomy is stalled meantime the years, however after an intensive study on phylogeney of 113 species of the family Botryosphaeriaceae through ribosomal DNA sequences *Macrophomina* and Tiarosporella has been separated as two different genera (Crous et al., 2006).

Classification of *M. phaseolina*

Kingdom:	Fungi
Division	Ascomycota
Class	Dothideomycetes
Order	Botryosphaeriales
Family	Botryosphaeriaceae
Genus	Macrophomina
Specie	*M.phaseolina*

(Goidanich, 1947) examined the original material of ***M. phaseoli*** collected by Tassi and renamed it as ***M. phaseolina*** (Tassi) Goid. Rhizoctonia is predominantly found in subterranean habitat both as parasite and saprophyte. Soil-borne pathogens are especially challenging because their infection propagules often survive for several years in the soil and they reduce yield and quality in numerous crops. Soil-borne diseases are difficult to detect, predict and manage because the soil matrix is complex, making it difficult to understand the variables that govern infection processes and epidemics.

M. phaseolina, the causal agent of charcoal rot is a soil- and seed-borne polyphagous pathogen. It causes diseases of more than 700 crops and non-crop species, including economically important hosts such as soybean, sunflower, corn, sorghum, cowpea, peanut and cotton (Csöndes et al., 2012; Ijaz et al., 2013; Ndiaye et al., 2010) and also causes non crop (human disease). Besides being an opportunistic plant pathogen, several clinical reports have also established ***M. phaseolina*** as an intermittent human pathogen that may cause cutaneous and several fungal infections (Srinivasan et al., 2009; D. H. Tan et al., 2008). ***M. phaseolina*** has the potential to neutralize plant, animal, as well as human immunity in immune-suppressed patients undergoing prophylactic antifungal treatments (Arora, Dilbaghi, & Chaudhury, 2012). For successful development of charcoal rot disease of sunflower, virulent isolate of ***M. phaseolina*** accompanied by low moisture and high temperature are essentially. The severity of infection depends upon relative humidity, temperature, nature of isolate, climatic region, and host cultivar (Ahmad, 1996). Important diseases caused by ***M. phaseolina*** include color rot, damping off, charcoal rot, stem rot, root rot, and seedling blight are in economically important crops (Babu, Saxena, Srivastava, & Arora, 2007). The plants affected with fungus show necrotic lesion on different parts such as branches, peduncles, and stems. A higher temperature and low moisture favors the disease development (Aegerter, Gordon, & Davis, 2000).

The microsclerotia of the pathogen can survive on infected plant debris and soil for a long period, 2–15 years depending on the environmental conditions (R. E. Baird, Watson, & Scruggs, 2003; Vasebi, Safaie, & Alizadeh, 2013). Microsclerotia are usually spherical, black, and oblong. However, there is a great variation in their shape and size which are depending on substrate, isolates, and temperature. M. phaseolina (Tassi) Goid. is a soilborne plant pathogen belonging to the phylum Deuteromycetes and class Coelomycetes. It is highly variable, with isolates differing in microsclerotial size and presence or absence of pycnidia. The pycnidial stage is not common on soybean, but is on peanut. Pycnida are initially immersed in host tissue, then erumpent at maturity. They are 100-200 μm in diameter, dark to grayish, becoming black with age. The pycnida bear simple, rod-shaped conidiophores, 10-15 μm long. Conidia (14-33 x 6-12 μm) are single celled, hyaline, and elliptic or oval (O. D. Dhingra & Sinclair, 1978). Colonies in culture range in color from white to brown or gray and darken with age. Hyphal branches generally form at right angles to parent hyphae, but branching is also common at acute angles. Aerial mycelium with completely or partially appressed growth may or may not be produced in culture. Some isolates may form concentric growth rings (O. D. Dhingra & Sinclair, 1978).

2.1.1 Symptoms

M. phaseolina is able to cause different symptoms: hollow stem rot, wilt, and preemergent and post-emergent damping off, depending on the plant tissue colonized. In the case of melon, ***M. phaseolina*** infects plant roots and later colonizes the fruit via the peduncle through the abscission zone. Once the fruit is colonized, *M. phaseolina* infects the seed coat and cotyledons, and these infected seeds give rise to diseased seedlings that can increase the inoculum potential in the soil (Reuveni, and, & Krikun., 1983). Predominant symptoms of charcoal rot on sunflower are gray to black discoloration with lesions on the stem above the soil line, black microsclerotia usually observed in the fibro-vascular system of roots

and lower internodes covering an average of one-third of the plant height (Khan, 2007; Raut, 1985). Disease symptoms are clearly visible from the time of emergence and can be evaluated at various stages of development of the plant. M. phaseolina can infect roots which show necrotic lesions (Adam, 1986; D. Bouhot, 1967). On adult plants, M. phaseolina causes lesions on stem, spike and seed. On stems, lesions are light brown and appear at the ramification point of the lateral secondary branches. Colonized tissues become gray and covered with abundant minute black punctuations. Initially these punctuations are immersed, becoming gradually more prominent. The most striking symptom is the sudden wilting and drying of the whole plant while most of the leaves remain green. The stem and branches are then covered with black bodies and give the charcoal or ashy appearance of dead plants. Withering can be observed from seedling to mature stage and is the result of necrosis of roots, stems and mechanical plugging of xylem vessels by microsclerotia, but also by toxin production, and enzymatic action (Kuti, Schading, Latigo, & Bradford, 1997). Charcoal rot is characterized by lodging of plants as they approach maturity. Lodging is due to the weakened condition of the stalk because of disintegration of the pith and cortex by the pathogen, leaving the lignified fibro-vascular bundles suspended as separate strands in the hollow stalk. The vascular bundles are profusely covered with tiny black sclerotia of the pathogen, which give the charcoal appearance to the affected area. Thus, the name“Charcoal rot” describes the appearance of the disease inside the infected roots and stalks. In human infections *M. phaseolina* hyphaehave skin lesions and purulent dischargeof immunosuppressive patients, nausea, vomiting, watering and swelling in the eye (Srinivasan et al., 2009; D. H. Tan et al., 2008).Thus *M. phaseolina* might be considered significant to both plant and human health.

2.1.2. Disease cycle

M. phaseolina produces sclerotia in root and stem tissues of its hosts that enable it to survive adverse environmental conditions (G. E. Cook, M.G. Boosalis, and, & Odvondny, 1973; G. Short et al., 1980). In PDA, pycnidia are not produced except under some specific incubation conditions (Gaetán, Fernandez, & Madia, 2006) and only some times in host crops (J. D. a. Mihail & Taylor., 1995), and their importance in the epidemiology of the fungus likely depends on the host involved as well as the fungal isolate (Ahmed & Ahmed., 1969). On cowpea, pycnidia are produced at the end of the rainy season, but their epidemiological significance seems minor. On the contrary, in jute crops. Microsclerotia in soil, infected seeds or host tissues serve as primary inoculum (Abawi, 1990; D Bouhot, 1968; O. D. Dhingra & Sinclair, 1978). Root exudates induce germination of microsclerotia and root infection of hosts. The infective hyphae enter into the plant through root epidermal cells or wounds. During the initial stages of pathogenesis, the mycelium penetrates the root epidermis and is restricted primarily to the intercellular spaces of the cortex of the primary roots. As a result, adjacent cells collapse and heavily infected plantlets may die. At flower onset, the fungal hyphae grow intracellularly through the xylem and form microsclerotia that plug the vessels (Mayek, R. G. Espinosa, C. L. Castañeda, and, & Simpson., 2002) and disrupt host cells. The infected plants show necrotic lesions on stems, branches and peduncles. From pod peduncles, the fungus spreads to the pods and invades grains. Heavily infected plants die prematurely due to the production of fungal toxins like phaseolinone (Bhattacharya, Dhar, Siddiqui, & Ali, 1994) and production of fungal tissue that plugs the host vessels. In soybean, formation of microsclerotia is conditioned by flowering and pod setting (Wyllie & Calvert, 1969) and may be indicative of initiation of death of the host (G. Short, Wyllie, & Ammon, 1978). After plant death, colonization by mycelia and formation of sclerotia in host tissue continues until tissues are dry. The mycelium and microsclerotia produced in infected plant material, including plant residues, are the means of propagation of

the pathogen. Microsclerotia in soil, host root and stems are the main surviving propagules. After decay of root and plant debris, microsclerotia are released into the soil. They are distributed generally in clusters at the soil surface and are localized mainly at a depth of 0-20 cm (Campbell & V.Gaag., 1993). They can survive for 15 years depending on environmental conditions and whether or not the sclerotia are associated with host residue (R. E. Baird et al., 2003). Factors that adversely affect the survival of these propagules includes repeated freezing and thawing of soil, low carbon: nitrogen ratios in soil and soil moisture content (O. Dhingra & Sinclair, 1975).

2.1.3.Distribution

The charcoal root rot organism, ***M. phaseolina*** is one of the most damaging pathogens in arid region and has a wide host range (Waller, 1976). In Sri Lanka Small 1927) In India, it has been reported from Andhra Pradesh, Bihar, Gujarat, Karnataka, Maharashtra, Madhya Pradesh, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal (Uppal, 1931; Likhite and Kulkarni, 1934; Deshpande et al., 1969 and Philip et al., 1969). Dry root rot caused by *R. bataticola* has been reported from Australia, Ethiopia, India, Iran, Lebanon, Syria and United States (Nene, 1978). Uruguay, and Yugoslavia in (1966) Argentina and Senegal (1967), Hungary (1970), France, (1976), Egypt (1980) and Pakistan, 1982 (Bhutta, 1998) North America (Pratt, McLaughlin, Pederson, & Rowe, 1998). Western Australia (Khangura & Aberra, 2009) West Africa (Msikita, James, Wilkinson, & Juba, 1998). Georgia, USA (R. Baird & Brock, 1999). Honduras (Bruton & Miller, 1997). China (J. Zhang, Zhu, Duan, Wang, & Li, 2011). Iran (Razavi & Pahlavani, 2004). Florida (Iriarte et al., 2007). Iowa (Yang & Navi, 2005). North Dakota (Bradley & Río, 2003). Minnesota (ElAraby, Kurle, & Stetina, 2003). Northwestern Argentina (Baino, Salazar, Ramallo, & Kirschbaum, 2011). France (Baudry & Morzieres, 1992). Spain (Avilés et al., 2008). California (Koike et al., 2009). Turkey (Mahmoud & Budak, 2011).Iraq (Hassan & Ahmed, 2015).

2.1.4.Host Range

Charcoal rot disease in world has a wide hosts range and is a serious threat especially in tropical and subtropical regions with arid to semi arid climates in Africa, Asia, Europe and North and South America. The pathogen infects over 500 plant species and has a wide geographic distribution. Major cultivated hosts include: sunflower (*Helianthus annuus*) ,Arachis hypogaea (peanut), Beta vulgarius (beet), *Brassica oleracea* (cabbage), *Capsicum annuum* (pepper), *Cicer arietinum* (chickpea), *Citrus spp. Corchorus sp., Cucumis sp., Fargaria sp., Glycine max* (soybean), *Gossypium sp., Ipomoea batatas* (sweet potato), *Medicago sativa* (alfalfa), *Phaseolus sp., Pinus sp., Prunus sp., Sesamum indicum* (sesame), *Solanum tuberosum* (potato), *Sorghum bicolor*(sorghum), *Vigna unguiculata* (bean) and *Zea mays* (corn) (Reuveni et al., 1983). pepper (*Capsicum annum* L.), papaya (*Carica papaya* L.), sweet orange (*Citrus sinensis* L. Osbeck), coffee (*Coffea arabica* L.), soybean (*Glycine max* L.) Merr) sunflower (*Helianthus annus* L.),msweet potato (*Ipomoea batatas* L) Lam.) apple (*Malus domestica* Borkh.), alfalfa (*Medicago sativa* L) plantain (*Musa paradisiaca* L.), tobacco (*Nicotiana tabacum* L.), common bean (*Phaseolus vulgaris* L.), garden pea (Pisum sativum L.), tomato (*Lycopersicon sculentum* Mill.), potato (*Solanum tuberosum* L.), grainsorghum (*Sorghum bicolor* L.) Moench), cacao (*Theobroma cacao* L.), clover (*Trifolium sp*.), grape (*Vitis vinifera* L.), and corn (*Zea mays* L.) (Reichert and Hellinger 1947).

The charcoal rot disease caused by ***M. phaseolina*** is one of the most destructive pathogens in arid regions and has wide host range (Waller, 1976). Charcoal rot due to R. bataticola has been reported in Austria, Ethiopia, India, and Turkey (Nene, 1978), on chilli (Mathur and Gurjar, 1999; Bunker and Mathur, 2001) and on urdbean (John et al., 2008). M. phaseolina infection on sunflower was first reported from Sri Lanka by (Small 1927) Later on it was reported from Uruguay, Australia and Yugoslavia in (1966) Argentina and Senegal (1967), Hungary (1970), USA(1971),India (1973), France, (1976), Egypt (1980) and

Pakistan, 1982 (Bhutta, 1998). In Turkey, Eskişehir province by (Mahmoud & Budak, 2011) disease was identified and reported.

2.1.5. Morphological and molecular identification of *M.phaseolina*

Identification of an organism is key to its classification and taxonomy. In the past, fungi and other organisms have been identified based on their morphological characteristics. The Saccardo system was primarily based on morphology of sporulation structures as they are known in nature as well as the morphology and pigmentation of conidia and conidiophores (Barnett & Hunter, 1972). The morphological features used to identify fungi may vary from organism to organism but largely depend on their characteristics in culture. *M. phaseolina* isolates on PDA are characteristically grey to black in color, They produce sclerotia from specialized highly compressed hyphae cells called monoloids just like . The color of the mycelial mat, and the size and color of sclerotia are also used to morphologically identify *M. phaseolina* (Aboshosha, Attaalla, El-Korany, & El-Argawy, 2007; Beas-Fernandez, De Santiago-De Santiago, Hernandez-Delgado, & Mayek-Perez, 2006). Recently, modern techniques of DNA analysis have now been developed for faster and more specific identification of organisms.

In recent years, some species-specific molecular diagnostic tools have been developed to broadly identify the organisms. Conventional polymerase chain reaction (PCR) markers based on the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) were reported by (Babu et al., 2007). the application of (PCR) is used widely used in diagnostic labs and clinics in both plant and medical sciences for specific detect the organism(Cooley, 1992; White, Bruns, Lee, & Taylor, 1990). Advances in molecular techniques have provided alternative techniques for the reliable identification of fungi. Internal transcribed spacers (ITS) and 18S rRNA have been one of the most conserved genes used to identify the fungus (Babu et al., 2010) . Species-specific primer sets were developed from a sequence characterized amplified region for use with both SYBR green and

TaqMan quantitative PCR (qPCR) assays for pathogen detection in soil and infected plants (Babu, Mesapogu, Sharma, Somasani, & Arora, 2011). In addition, a loop mediated isothermal amplification assay was developed using the ITS region of *M. phaseolina* to quickly detect the fungus within infected soybean plants (Lu, Song, Zhang, Wang, & Zheng, 2015) . Sanger sequencing is another common molecular method that is usually employed when a deeper analysis of DNA is required(Pereira, Carneiro, & Amorim, 2008). Sanger sequencing of DNA enables analysis of DNA for several projects such as identification of homologous genes across species or identification of mutations or merely identification of individual species for determination of relationships among organisms. Identification of species in Sanger sequencing is achieved by performing a BLAST search of the sequence obtained in vast GenBank sequence database. in order to genotype different isolates of *M. phaseolina* while Purkayastha, Kaur, Dilbaghi, and Chaudhury (2006) demonstrated the use of PCR-based markers targeted to the rDNA ITS region to characterize different populations of *M. phaseolina* . Baird et al.(2010) and Arias et al. (2011) reported on SSR markers. Despite the general perception that *M. phaseolina* is a broad hostrange pathogen, there have been reports in the literature suggesting host specialization of isolates. The ITS region has been demonstrated to selectively detect several agriculturally important fungi. Based on this discovery, Babu et al. (2007) analyzed sequences of M. phaseolina from isolates and from GenBank, and designed *M. phaseolina* specific primer probe (MpKH1) from the conserved region, adjacent to the 5.8 S gene. Using the specific primers a forward (MpKFI) and reverse (MpKRI), M. phaseolina can be identified by its characteristic bands of between 300 to 400 bp. Genetic and pathogenic variation in *M. phaseolina* strains have been assessed in numerous isolates but so far, it is hard to discriminate of *M. phaseolina* isolates from exact hosts or geographic locations due to heterogeneous nature. Lack of a strong association between the geographical origin and genotype propose high diversity among *M. phaseolina* strains (Jana, Singh, Koundal, & Sharma, 2005).

2.2. *Fusarium* pathogens

The *Fusarium* genus is one of the utmost complex and adaptive species in the Eumycota and the *Fusarium oxysporum* species complex includes plant, animal and human pathogens and a diverse range of non-pathogens (Gordon, 2017). Members of *Fusarium* species are ubiquitous soil-borne pathogens of a wide range of horticultural and food crops which cause destructive vascular wilts, rots, and damping-off diseases (Bodah, 2017). In addition to the losses caused before or during harvest, some *Fusarium* species are capable of producing mycotoxins in food and agricultural commodities (Mudili et al., 2014). *Fusarium* toxins are the most abundant natural contaminants of diets containing cereals and other grains(Naveen K Kalagatur et al., 2015; M. Kumar, Kothari, & Gupta, 2016). and suspected to be implicated in numerous diseases among mammals and other living beings (Naveen Kumar Kalagatur, Kamasani, & Mudili, 2018). The fumonisins, belong to the family of food-borne carcinogenic mycotoxins, with reports of toxic activity of Fo strains isolated from various products that exhibited different degrees of toxicity to experimental animals (Venkataramana et al., 2012). *F oxysporum* included pathogenic fungi. It includes strain which were pathogenic to both human and plants. *F.oxysporum* contained more than 120 different formae specials with a wide host range that included most of grasses and trees (Daboussi & Langin, 1994).

2.2.1.Ecology and Epidemiology of *F. oxysporum*

The epidemiology of root-infecting fungi in the soil is complex and factors, such as inoculum density, pathotype, plant age, host resistance and its genetic potential, air and soil temperature, soil moisture, soil nutrients, plant density and other cultural practices, may affect wilt development (Haware & Nene, 1980). *F. oxysporum* is able to survive in the soil for a long period by forming resting spores, thick-walled propagative structures, known as chlamydospores, and dormant mycelium in infected plant debris as well as survive in soil either in

dormant form or saprophytically without a suitable host for several years (Taylor, Taylor, Lindbeck, Chen, & Ford, 2016) and is capable of colonizing residues and roots of most crops grown in rotation with lentil. The causal pathogen is both seed- and soilborne and can survive in soil, even in the absence of its host for six years (Ayyub, Khan, Ahmad, & Iftikhar, 2003). Being soil-borne pathogen of crops worldwide, it may cause severe damage and complete crop failure under favorable conditions for infection by the pathogen and disease development (Chaudhary & Kaur, 2002) .

2.2.2.*Fusarium* as Animal and Human Pathogen

Certain sp of *Fusarium* had also been observed to be an emerging human pathogen in immune compromised patients patients (Vartivarian, Anaissie, & Bodey, 1993). *F oxysporum* included pathogenic fungi. It includes strain which were pathogenic to both human and plants. *Fusarium* species can cause various types of infection to humans including superficial infection such as keratitis and onychomycosis, locally invasive, or disseminated infections and may also cause allergic such as sinusitis. *Fusarium* that causes human mycoses usually exists as saprophyte in the environment (D. P. Short, O'Donnell, Zhang, Juba, & Geiser, 2011). Approximately 15 species of *Fusarium* have been reported to be associated with human and animal pathogens including *F. solani, F. oxysporum, F. verticillioides, F. proliferatum* and *F. anthophilum* (Jain et al., 2011). Indicated that *F. falciforme*, a phylogenetic types in the *F. solani* types complex is the most common species related with fungal keratitis which is found in soil and plant debris, and considered as the prime source of infection (N. Zhang et al., 2006). Diseases caused by *Fusarium* in animal are parallel to diseases that infect human. *Fusarium solani* was reported causing immune competent diseases of mice and causing degradation of exoskeleton of shrimps and turtles (Brown & Proctor, 2013). Other *Fusarium* species such as *F. moniliforme*, *F. oxysporum* as well as *F.*

solani have been reported causing black gill disease of kuruma prawn (Penaeus japonicas) in Japan (Hatai, 2012).

2.3. *Trichoderma spp*

Trichoderma spp (syn. Hypocrea), are soil borne, green spored ascomycetes that are present in nearly all types of temperate and tropical soils. They have various characteristics and applications that allow them to be successful colonisers of their habitats, efficiently fighting their competitors and establishing a predominant role in their environment (Felix, Noronha, & and Miller, 2014). The first description of a fungus under the genus Trichoderma dates back to 1794 (Persoon, 1794) and in 1865 a link to the sexual state, *Hypocrea*, was suggested by Tulasne and Tulasne (1865). This anamorph–teleomorph relationship was only confirmed more than 100 years later for *Trichoderma* reesei and *Hypocrea jecorina* (Kuhls et al., 1996). *Trichoderma* is a well-studied genus of fungi that currently comprises of more than 200 genetically defined species (Atanasova et al., 2013). *Trichoderma* taxa are morphologically very similar and for many years, they have been considered as a single species. In 1969, new species were discovered and Rifai (1969) proposed a consolidated taxonomical scheme and defined nine morphological species aggregates. Mycoparasitic species of Trichoderma, able to antagonise commercially important plant pathogens, were classified as Trichoderma harzianum (I. S. Druzhinina, Kopchinskiy, & Kubicek, 2006).

Great genetic diversity found among *Trichoderma harzianum* isolates,which now have their own species identifications such as *T. hamatum, T. harzianum, T. viride, T. aureoviride, T. virens, T. citrinoviride, T. roseeii, T. crissum, T. longibrachiatum, T. pseudokoningii, T. ovalisporum, T. koningii, T. asperellum, T. polysporum and T. saturnisporum.* There are four distinct species within the *Trichoderma harzianum* aggregate: *T. harzianum, T. atroviride, T. longibrachiatum*, and *T. asperellum* (Hermosa et al., 2000). There appears to be a consensus as to the most prevalent species isolated from the rhizosphere of crop

plants with *T. harzianum*, *T. hamatum, T. atroviride*, and *T. viride* being the most isolated *Trichoderma* species (Kredics et al., 2014).

2.3.1.Classification of *Trichoderma*

Classification of *Trichoderma* as explained by (Samuels, 1996);

Kingdom: Fungi
Division: Ascomycota
Subdivision: Pezizomycotina
Class: Sordariomycetes
Order: Hypocreales
Family: Hypocreaceae
Genus: *Trichoderma*

The taxonomy of *Trichoderma* was first described by Persoon in his classification of fungi in 1794. Unfortunately, his classification of *Trichoderma* was problematic where his observation included other fungi such as Puccinia, Mucor, Ascobolus and some slime molds such as Physarum, Trichia and Stemonitis (D. Klein & Eveleigh, 2002).

In 1939, Bisby Propose dthat *Trichoderma* consists of a single species, *T. viride*. This concept led to nearly all strains of *Trichoderma* was identified as "*T. viride*" in literatures before 1969. Therefore, most of the taxa determined before 1969 are probably misidentified since *T. viride* is a relatively rare species (I. Druzhinina & Kubicek, 2005).

In 1969, Rifai proposed the concept of "aggregate" species, where *Trichoderma* species are divided into nine "species aggregates", namely T. *aureoviride Rifai, T. hamatum Bain, T. harzianum Rifai, T. koningii Oudem, T. longibrachiatum Rifai, T. piluliferum Rifai, T. polysporum Rifai, T. pseudokoningii Rifai and T. viride*. However, Rifai admitted that each species aggregate was likely

to contain more than one morphologically indistinguishable species. Starting from 1984, Bissett started to revise Rifai's aggregate species. In 1991, Bissett discussed the difficulty to distinguish *Trichoderma* species based on Rifai's species aggregates, since only five of Rifai's aggregates species (*T. harzianum, T. longibrachiatum, T. pseudokoningii, T. piluliferum and T. polysporum*) were narrowly defined, while other aggregates were having relatively large number of species (Chaverri & Samuels, 2004). In the same year, Bissett subdivided the genus into five sections, which are Longibrachiatum, Trichoderma, Pachybasium, Saturnisporum and Hypocreanum (I. a. Druzhinina & Kubicek, 2004).

2.3.2.Morphological of *Trichoderma*

Morphological description of *Trichoderma* is not easy task. The useful morphological characters, such as colour or pattern is quite limited for defining different species of *Trichoderma*. The different useful characters in distinguishing Trichoderma species can be colony character, growth rates in culture, conidia production, pigments, odours, conidiophore branching and aggregation . Some species grow with distinctable colony characters, hence it is not easy to sufficiently describe them all and therefor being useful for identifaction. Different species exhibit usually different growth rates and are also distinguishable by the way they produce conidiophores (Bissett, 1991; Samuels et al., 2006). Some do produce conidia from effused conidiophores and some do it from conidiophore aggregated in pustules or fascicles. Some species can be described by their diffusable pigments, like the strains of section Longibrachiatum producing bright greenish . Characters useful for characterization and identification in other Hyphomycete genera frequently are not as useful in differentiating *Trichoderma* species, usually because of the narrow range of variation of the simplified morphology in *Trichoderma*, or because descriptive terms to describe variation in colour or pattern are not sufficiently precise to define differences between species (W Gams, Bissett, & MALIK, 2002). strains often can be readily identified by a distinctive

morphology that includes rapid growth, bright green or white conidial pigments, and a repetitively branched, but otherwise poorly defined conidiophore structure(Walter Gams, 1971).

Surface has been accepted as designating the appearance of the aerial portion of the colonies. This is one of the most stable characters when the same medium and conditions are used. Although successive cultures of the same organisms may show some variation in color, zonation, or character of the margin, they are differences entirely of degree and not of kind. The type of growth therefore, maybe considered a reliable diagnostic character (Thom, 1910).

Conidiophores: Data as to conidiophores include the length, septation, markings, and habit of growth, whether arising directly from the substratum or as branches of aerial or submerged mycelium. While considerable variation in the length of conidiophores will often occur, even in the same culture, the majority will approach an average length.

Conidia: The characters of the conidia may be used as almost the sole bases for classifying species. With the possible exception of markings of the conidial wall shown by some species, these characters are constant and marked variations from the mode are rare, Conidia are much more constant in size than conidiophores (Welles, 1925).

2.3.3.Molecular diagnosis of *Trichoderma*

Recently, Molecular tools like DNA sequencing had their great breakthrough in the mid 90s and made available what was missing for so long – a great variety or number of characters applicable for the description of the taxa and their phylogeny (Kubicek, Bissett, Druzhinina, Kullnig-Gradinger, & Szakacs, 2003). Molecular techniques the morphology-based taxonomy of *Trichoderma* was reevaluated. The molecular markers used in the study of *Trichoderma* taxonomy include protein markers (isozyme analysis) and DNA markers. The strategies to identify *Trichoderma* using DNA markers are sequence analysis of internal

transcribed spacer (ITS) region, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), and chromosome and karyotyping analysis (Lieckfeldt, Kuhls, & Muthumeenakshi, 2002). In recent years, the development of TrichOKEY and TrichoBLAST facilitate the identification of *Trichoderma* based on oligonucleotide DNA barcode. TrichOKEY is a program used to identify *Trichoderma* based on several genus-specific hallmarks located within the ITS1 and ITS2 sequences (I. S. Druzhinina et al., 2005). TrichoBLAST is a database supported by sequence diagnosis and similarity search tools based on those frequently used phylogenetic markers, including ITS1 and 2, intron tef1_int4 and intron tef1_int5 (Kopchinskiy, Komoń, Kubicek, & Druzhinina, 2005). International Submission on *Trichoderma* Taxonomy listed 104 *Trichoderma* species which have been characterized at molecular level (S. H. Tan, 2013).

2.3.4.Economic Importance of *Trichoderma*

Trichoderma can work as bio-control agents in various ways, It tends to grow faster or use its food source more efficiently than the pathogen, thereby crowding out the pathogen and taking over; this is known as nutrient competition.They may excrete a compound that can either slows down or completely inhibit the growth of pathogens in the surrounding area of such a compound called antibiosis. It may feed on or as direct pathogenic species directly known as parasitism. It may induce a plant to produce a chemical that protects it from the pathogen, which is induced resistance. They can grow in an endophytic way in other species and supports plant growth.(Noor, 2017).

2.3.5.Mechanisms of *Trichoderma Spp*

There are sundry bio mechanisms of *Trichoderma spp* against pathogens, as mycoparasitism, antibiosis, inactivation of the pathogen enzymes, production of Lytic enzymes,competition with pathogens, solubilization and sequestration of

inorganic nutrients and secretion of secondary metabolites (F Vinale et al., 2006), and also *Trichoderma spp* are good antagonists against pathogenic fungi. They are able to survive under extreme competitive conditions (Benítez, Rincón, Limón, & Codon, 2004).

2.3.5.1. Mycoparasitism

Mycoparasitism is considered an important mechanism of biological control and probably depends on the production of lytic enzymes including β-1,3-gluconase, and proteases (Haran, Schickler, & Chet, 1996). Several chitinolytic enzymes have been reported in *T. harzianum* including endochitinases, exochitinases and 1, 4-β-N-acetylglucosaminidases which are induced during growth in liquid medium containing chitin as carbon source(Haran, Schickler, Oppenheim, & Chet, 1996), and also mycoparasitism is a complex process including several steps. The initial interaction shows that the hypha of the mycoparasites grows directly towards its host (Chet, Harman, & Baker, 1981). When the mycoparasite reaches the host, its hypha coils it or attaches to it by forming a hook-like structure. Following these interactions hypha sometimes penetrates the host mycelium, apparently, by partially degrading its cell wall (Elad & Chet, 1983).

2.3.5.2. Antibiosis

The importance of antibiotics for biocontrol activity was demonstrated in several studies. Dennis and Webster (1971) found that many *Trichoderma* strains produced volatile and nonvolatile antibiotics. Several secondary metabolites produced by *Trichoderma spp* are known, These metabolites are most likely produced with the purpose of increasing the competitiveness of the producing Trichoderma strain towards other microorganisms (Szekeres et al., 2005). Howell (2003) isolated and described a new antibiotic, gliovirin, from *T. virens* that was strongly inhibitory to *Pythium ultimum* and *Phytophthora* species. *Trichoderma*

species have also been reported to produce antimicrobials among their secondary metabolites (Francesco Vinale et al., 2008).

Trichoderma spp. produces 43 substances that have antibiotic activity (Sivasithamparam & Ghisalberti, 2002), like pyrones, isonitriles, polyketides, peptaibols, dikeyopiperazines, sesquiterpenes, and steroids have been associated with biocontrol activity of some species and strains of *Trichoderma* (Howell, 1998).

2.3.5.3.Inactivation of the Pathogen Enzymes

Enzymes such as chitinases and /or glucanases produced by the biocontrol agent are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitin, and β- glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity (Howell, 2003). *T. harzianum* (T39) produce proteases that are capable of degrading the pathogens plant cell wall degrading enzymes, and thereby reducing the ability of the pathogen to infect the plant. They also showed that protease may be directly toxic to germination of the pathogen and also may inactivate its enzymes (Elad & Kapat, 1999).

2.3.5.4.Lytic enzymes

One reason for the astonishingly remarkable fast and efficient exploitation of almost any organic compound and therefor nutrients of *Trichoderma* species, is their enormous equipment of lytic enzymes. Organic matters consist of a wide range of sugars, homo- and heteropolysaccharides. Proteases do further help to damage other microorganisms which could compete with them. Several hydrolytic enzymes of different antagonistic *Trichoderma* species have been cloned and their role and regulation in mycoparasitism was characterized (Scherm, 2008). Chitin and β-1,3-glucan are the principal modules fungal cell walls, *Trichoderma* possess a range of chitinases and β-1,3-glucanases which do the main work in the

degradation of the latter (Mahadevan & Tatum, 1967). It seems that - after breaking down the obstacle of the pathogens' cell wall – *Trichoderma spp*. are able to prevent the pathogen from germination or further growth by the release of β-1,3-glucanases that collaborate with chitinases and antibiotics (Benítez et al., 2004). Some of the β-1,3-glucanases have been isolated and cloned so far, as bgn13.1(De La Cruz, Pintor-Toro, Benitez, Llobell, & Romero, 1995) and lam1.3 (Cohen-Kupiec, Broglie, Friesem, Broglie, & Chet, 1999) from *H. lixii*, glu78 from *H. atroviridis* (Donzelli & Harman, 2001) and Tv-bgn1 and Tv-bgn2 from *H. Virens* (Kim, Baek, Uribe, Kenerley, & Cook, 2002) . However an effective antagonist needs further glucanases to degrade other structurally important cell wall components like β-1.6-glucan, α-1,3-glucan, or faced with chitin as only carbon source. So far three β-1.6-glucanases from *H. lixii* (BGN16.1, BGN16.2, BGN16.3) have been purified and are believed to be essential in the fungal cell wall attack, rich of chitin (Montero, Sanz, Rey, Monte, & Llobell, 2005). The working object of α-1,3-glucanases are glucose polymers linked by a 1.3-glucosidic bounds. The α-1,3-glucanases can be divided into endo- (final degradation product is a small glucose polymer) and exo-glucanases (final degradation product is a glucose monomer). Studies on exo- α-1,3-glucanases agn13.1 and agn13.2 from *H. lixii* proved their role in mycoparasitism (Sanz, Montero, Redondo, Llobell, & Monte, 2005).

Proteases. Several different extracellular proteases have been isolated from *Trichoderma* (Suarez, Rey, Castillo, Monte, & Llobell, 2004; Williams, Clarkson, Mills, & Cooper, 2003). They seem to be another important set of enzymes enabling a wellorganised biocontrol reaction. It was shown that proteases of *H. lixii* could deactivate to some extend hydrolytic enzymes released from pathogens, resulting in reduced germination activity by the pathogen (Elad & Kapat, 1999; Kapat, Zimand, & Elad, 1998). Besides deactivation of plant pathogens' enzymes, proteases may be important for the mycoparasitic process by degrading the protein components of the host well wall. In order to better

understand the way of reaction and involvment of protease in mycoparasitic interaction some proteases of *Trichoderma spp*. have been cloned. Prb1 protease of H. atroviridis, like its orthologues from *H. virens* and *T. hamatum* (Pozo, Baek, Garcıa, & Kenerley, 2004), is a subtilisin-like serine protease and was shown to play its part in mycoparasitism of *R. solani* (Cortes et al., 1998; Olmedo-Monfil, Mendoza-Mendoza, Gomez, Cortes, & Herrera-Estrella, 2002).

2.3.5.5.Competition

Competition occurs when more of one organisms require the same resource and the use of this by one reduces the amount available to the other. The nutrient sources in the soil and rhizosphere are frequently not sufficient for microorganisms and starvation is the most common cause of death for microorganisms. For a successful colonization of phyllosphere and rhizosphere a microbe must effectively compete for the available nutrients. There is a general believe that competition between pathogens and non-pathogens for nutrient resources is an important issue in biocontrol. It is also believed that competition is more critical for soil borne pathogens, including *M.phaseolina,,Fusarium* and *Pythium* species (Elad, Barak, & Chet, 1984; Loper & Buyer, 1991). Certain *Trichoderma* produce highly efficient siderophores that chelate iron and stop the growth of other fung (Chet & Inbar, 1994). In addition, *Trichoderma harzianum* controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, and become more effective when the nutrient concentration reduces (R. J. Cook, Papavizas, & Tjamos, 1992).

The efficient use of available nutrients is based on the ability of *Trichoderma spp* to obtain energy from the metabolism of different sugars, such as those derived from polymers, wide-spread in fungal environments including cellulose, glucan and chitin among others. *Trichoderma spp* strongly inhibits *R. solani*, *P. ultimum* and Chalara elegans when cultured in the same agar medium and the inhibitory action was associated with high rate and extent of CO2

accumulation in comparison with the plant pathogenic fungi (Ozbay & Newman, 2004).

2.4. *Dermatophytoses*

The dermatophytoses are among the most common human disease. Although the prevalence of those infections varies greatly, at least 10–20% of the world population may be infected with dermatophytes (Hryncewicz-Gwóźdź et al., 2011). The highly developed host-parasite relationship is responsible for a multitude of clinical manifestation and many of these host-parasite interactions depend upon specifc moieties and enzyme production, especially in *Trichophyton rubrum* which may enhance survival in tissues by chemically or physically altering the immediate environment or they may act directly by digesting host proteins, thus providing a source of nutrition (Liu et al., 2014).

The etiologic agents of the dermatophytoses are classified in three anamorphic (asexual or imperfect) genera, *Epidermophyton, Microsporum*, and *Trichophyton,* of anamorphic class Hyphomycetes of the Deuteromycota (Fungi Imperfecti). The descriptions of the genera essentially follow the classification scheme (Emmons, 1934), on the bases of conidial morphology and formation of conidia and are updated following the discovery of new species (Matsumoto & Ajello, 1987).The genera and their descriptions are as follows.

Epidermophyton spp. The genus *Epidermophyton* includes only 2 species. The colonies are slow-growing, powdery and unique brow- nish yellow in colour. This genus is devoid of micro co-nidia. Macro conidia are abundant and produced in clus-ters (Jagdish, 1995). These macro conidia are thin walled with smooth surface (Weitzman & Summerbell, 1995).

Figure 2.1. *Epidermophyton spp*.

Microsporum spp. The genus *Microsporum* includes 16 species. The colony morphology of *Microsporum* species on agar surface is either velvety or powdery with white to brown pigmen-tation (Jagdish, 1995). Both macro and micro conidia are produced but the predominant conidial structures are macro co-nidia. Micro conidia are less abundant. The macro co-nidia are multi septate with thick wall and rough surface, Rarely some species produce neither micro nor macro conidia(St-Germain & Summerbell, 1996).

Figure 2.2. *Microsporum spp*.

Trichophyton spp. The genus *Trichophyton* includes 24 species. The colonies on agar media are powdery, velvety or waxy. The predominant spore type is micro conidia with sparse macro conidia (Jagdish, 1995). Reverse side pigmentation is char-acteristic of the species and is used for the identification of the species within the genus (Wagner & Sohnle, 1995). The macro co-nidia are thin walled with smooth surface and variable shape. Some of the *Trichophyton* species are fas-tidious in their requirement for amino acid as nitrogen source. *Trichophyton tonsurans* requires ornithine, citrul-line and Arginine whereas *Trichophyton* mentagrophytes requires methionine. This nutritional speci-ficity has been used by many authors in the identifica-tion of the Trichoph yton species (Philpot, 1977; Weitzman & Summerbell, 1995).

Figure 2.3. ***Trichophyton spp***

2.4.1.Geographical Distribution

Geographically distributed dermatophytes, such as *Epidermophyton spp*. *Microsporum spp*. and *Trichophyton spp*. are multicultural. In these three genera *Trichophyton* has predominant causes than *Epidermophyton spp*. and *Microsporum* spp. However, in the genus *Trichopthyton, T. rubrum* is the basic causative pathogen than *T. tonsurans, T. verrucosum* and *T. mentagrophytes*. Consistent with the survey of the World Health Organization (WHO) on prevalence of dermatophytic infections, about 20% present, people have cutaneous infections worldwide. Tinea corporis is the most common fungal disease (about 70%) than Tinea as compared to *T. cruris, T. pedis* and Onychomycosis (Lakshmipathy & Kannabiran, 2010).Dermatophytic infections are found in all races and they are also common in all ages.

2.4.2.Pathogenesis

Through scars, wounds, injured skin and burns, dermatophytes may enter the host body. They can digest keratin as a source of nutrient so they invade non-living, upmost and keratinized skin layer that is stratum corneum and produce enzyme namely keratinase. At the site of infection, they cause inflammatory reaction and redness, swelling, heat or burning and alopecia are the signs of inflammatory reaction which are seen at the spot of infection. The pathogens stir away from the infection site due to inflammation and produce the ringed lesion. On the basis of disease and affecting organs pathogens are referred to as (Sultana, 2018)

Table 2.1. Fungal pathogens, diseases and affecting organs.

Fungal Pathogen	**Disease**	**Affecting Organ**
Trichophyton erinaceid	Ring-worm disease	Facial hair
T. violaceum	Black-dot	Nails
T. mannum	Ring-worm disease	Hand and palms area
T. tonsurans	Ring-worm disease	Scalps
T. rubrum	Jock itch	Groin area
T. spp.	Onychomycosis	Finger and toe nails
Microsporum gypseum	Tinea barbae	Face
M. Calais	Ring-worm disease	Arm, legs and trunk
Epidermophyton floccosum	Athlete's foot	Feet
E. floccosum	Tinea ungium	Nails
Cladiosporum carionii	Chromoblastomycosis	Hand, face, ear, neck and chest
Blastomyces dermatidis	Gilchrist's disease	Skin, lungs
Madurella spp.	Madura foot	Feet, hands
Hormodendrum spp.	Deep mycoses	Subcutaneous tissues

2.5. Importance of *Trichoderma* as Biological Control Against Plant and human disease.

Since almost 80 years the beneficial effects of *Trichoderma* species have been known and used in agriculture for fighting against soil-borne plant diseases (Weindling, 1934) and against human disease specially dermatophytes (Omero et al., 2004) .Knowledge became more and more detailed over these years, leading to the identification of several species particularly indicated in field application of disease control.

In a study by Bogumił, Paszt, Lisek, Trzciński, and Harbuzov (2013), A antagonistic activity against grey mould caused by Botrytis cinerea, are found that 52 isolates of *Trichoderma spp* had the ability to inhibit the mycelial growth of grey mould in in vitro conditions. resulting to The percentage reduction in the growth of Botrytis cinerea after six days of incubation at 25ºC ranged between 45 to78%.

Al-Obaidy and Al-Rijabo (2011) found that antagonism of 11 isolates of *Trichoderma spp*. against *M. phaseolina*, lead to increse the percentage inhibition in dual culture varied between 20.22 - 58.67%.

Sreedevi, Charitha Devi, and Saigopal (2011) confirmed in in their studies,effective *Trichoderma spp*. against the root rot caused by *M.phaseolina*. Five isolates of *Trichoderma spp*, lead to inhibition grow of fungi in dual culture which might ranged between 53.3 - 64.4%.

T. asperellum was appeared antagonistic influence against both *F. oxysporum* and *F. solani*, in which inhibition percentage of 66.67% and 65.52%, respectively (S. H. Tan, 2013) .

Verma, Kaur, Masih, Singh, and Singla (2017) found that *Trichoderma sp* and *T. viride* were inhibition of Fusarium disease, in which ratio inhibition reached 47.09 and 52.31% respectively.

Bastakoti, Belbase, Manandhar, and Arjyal (2017) found three of the *Trichoderma isolates* were inhibition of radial growth of *Sclerotium*

rolfsii,*Rhizoctonia solani*, *Fusarium solani* and *Sclerotionia sclerotiorum* in which reached 100 , 62 , 68 and 23 % respectively.

T.harzianum the great inhibiting against the *Trichophyton mentagrophytes*, *T. rubrum, T. schoenleinii, T. terrestre and T. verrucosum* fungi, in which the ratio of inhibition reached 100% at the concentration 2 mg/ml and *T.viride* was inhibited of growh the *Trichophyton mentagrophytes, T. rubrum, T. schoenleinii, T. terrestre and T. verrucosum* fungi, in which the ratio of inhibition ranged between 68.27-100% at the same concentration (Al-Obaidy & Al-Rijabo, 2011).

Aasi and Al-Aaraji (2018) Found that *T. harzianum* and their extract exhibited significantly high antifungal activity against *T. mentagrophyte* and *Microsporium. canis*. in which growth inhibition zones reched 14 and 12mm respectively at the concentration 0.5mg/ml

3. MATERIAL AND METHODS

3.1. Materials

3.1.1 Collection of Soil Samples

A total of 20 samples were collected from different collection sites in Adana. Twelve of them were soil, eight were compost. The soil samples were taken from a depth of 5 to 10 cm. The samples were collected in plastic bags, sealed in boxes and labeled with information of collection sites and origin of samples. Then, the samples were transported to the laboratory and processed within 24 hours.

3.1.2. Isolation of *Trichoderma spp* and *F. oxysporum*

Serial dilution technique was used to dilute the samples collected. The samples were homogenized and 5g of the samples were weighed and add to 100 ml distilled water. Thousand times (10^{-1}) and ten thousand times (10^{-4}) dilution of each sample was prepared. Then, 1000 µL of each diluted sample was pipetted onto potato dextrose agar medium (PDA). The inoculated PDA plates were then incubated at 28°C for 4 to 7 days. The plates were observed daily.Visible fungal colonies were transferred to new PDA plates and incubated at 28°C for 5 days.

3.1.3.Idintifiction of *Trichoderma* isolates and *F. oxysporum*

Trichoderma isolates were grown on PDA, incubated at 28°C for 7 days prior to microscopic identification. A small portion of specimen was sited on clean glass slide with 1- 2 drops of KOH solution, then the hyphae was gently dispersed. The slide was then observed under a light microscope with 400X magnification. Observations focused on the shapes and colours of spors, conidia, the branching patterns of conidiophores, and the appearance of chlamydospores.

3.1.4. Survey and collection of *M. Phaseolina* sample

An intensive survey was conducted during summer 2019 and 2020 on the incidence of charcoal rot in sunflower growing districts of Adana. Sunflower plants showing typical symptoms on stems bearing and characteristic symptoms of charcoal rot were collected from fields at different locations of Adana. Samples of 3–5 plants were sampled per field and brought to the laboratory. Isolates were given to name on the basis of genus and species name of ***M. phaseolina*** i.e. M.P

Table 3.1. Isolates of *Macrophomina phaseolina* collected from different locations of Adana.

S.No.	Name of District	Name of the Isolates
1	Kabasakal	M.P1
2	Kabasakal	M.P2
3	Balcalı	M.P3
4	Karaisalı	M.P4
5	Karaisalı	M.P5
6	Kozan	M.P6
7	Kozan yolu	M.P7

3.1.5. Isolation of *M. Phaseolina*

The plant samples infected were washed under running water to remove surface soil .Infected specimen cut into small pieces (0.5 -1 cm long), surface sterilized with 1 % sodium hypochlorite for 2 minutes and then rinsed twice in distilled water. The pieces were placed on Potato Dextrose Agar (PDA) medium containing antibiotics to avoid bacterial contamination. Inoculated petri dishes were incubated in dark at 25 ± 1°C for 2-3 days. For the fungus purification, a small portion of the fastest growing colony of *M. phaseolina* was taken from the periphery of 1 cm diameter petri dish and maintained on PDA slants by storing it under refridgeration (4°C). For the molecular identification the purified culture (0.5 cm disc) from isolate growing on PDA was transferred to patri dish and incubated

in dark at 25±1°C for 6 days, until the surface of PDA was covered. The patri dishs were stored at 4°C. until DNA extraction..

3.1.6.Dermatphytes

Trichophyton tonsurans and ***Microsporium canis*** specimens obtained from Prof.Dr.Mehmet Macit İLKİT. Çukurova University, Faculty of Medical. Departmant of Medical Mikrobiyology.

3.1.7. Potato Dextrose Agar (PDA)

PDA medium was used for culturing the fungus during this study. PDA was prepared according to the following recipe.

Potatoes	200g
Dextrose	20g
Agar	20g
Distilled water to make up the volume	1000 ml

3.1.8.Sabouraud's Dextrose Agar (SDA)

Sabouraud's dextrose agar media was employed for the culturing of Dermatophytes fungi. Composition of SDA.

Nutrient agar	20g
Peptone	10g
Glucose	40g
Distilled Water	1000 ml

3.1.9. Potato Dextrose Broth (PDB)

Potatoes, infusion from	200g
Dextrose	20g
Final pH	5.1g

3.1.10. Solutions preparation DNA Extraction for *M. phaseolina* and *Trichderma spp*

2X CTAB (Cetyl trimethyl ammonium bromide) to make 100 ml buffer

- 10 ml 1M Tris HCl
- 28 ml 5M Nacl
- 4 ml 0.5M EDTA
- 2 gr CTAB
- 50 ml distile water

Prepare 1 M Tris HCl solution (to make 100 ml solution)

- 12.11 g Tris HCl is added to 70 ml of distilled water. The pH is calibrated with HCl until 8. PH: 8 without tris insoluble.
- Final pH to 100 ml after pH adjustment

Preparation of 5 M NaCl solution (for making 100 ml solution)

- 29.22 g NaCl is dissolved in 70 ml distilled water and the final volume is completed to 100 ml

To prepare 0.5 M EDTA solution (to make 100 ml solution)

- 18.61 g of EDTA is added to 70 ml of distilled water. Sodium hydroxide (NaOH) is set to pH 8 with pellets. pH: 8 without EDTA insoluble.
- After pH adjustment, the final volume is completed to 100 ml.

Chelex 100 Prepared solution of 50ml Prepare a 5-10% by weight slurry of Chelex100 Resin (Biorad part 142- 1253,200-400 mesh Chelex, sodium form) and water (MilliQ) . The most effective way to do this is to take a 50 ml sterile falcon tube, place in on a scale inside a small beaker and zero the scale. Then add 5 grams of Chelex and fill to 50ml mark with water. Store at 20 – 25 °C

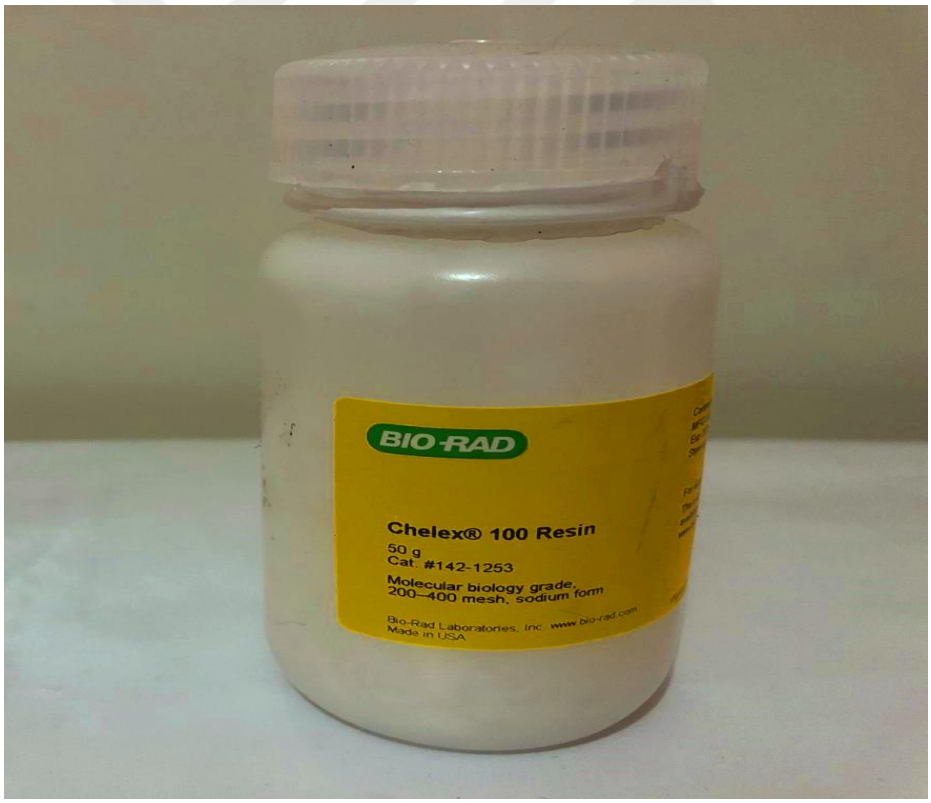

Figure 3.1. Chelex®100 Resin

TE Buffer Prepared solution of 2mL of 0.5M EDTA and 1M Tris HCl at pH 8.0 was dissolved in 800mL distilled water and final volume was adjusted to 1L.

3.2. Methods

3.2.1. Preparation of PDA medium

The medium was prepared by dissolving 39 g of PDA powder in 1000 ml distilled water and sterilized with autoclave at 121°C and pressure 15 for 15 minutes. After sterilization, the flask was left to cool. Before the medium hardened, the antibiotic Amoxicillin was added to 100 ml / l to prevent bacterial growth, then pour the medium into a petri dish.

3.2.2. Preparation of SDA medium

The medium was prepared by dissolving 65 g ofSDA powder in 1000 ml distilled water and sterilized with autoclave at 121°C and pressure 15 for 15 minutes. After sterilization, the flask was left to cool. Before the medium hardened, the antibiotic Amoxicillin was added to 100 ml / l to prevent bacterial growth, then pour the medium into a petri

3.2.3. Preparation of PDB medium

The medium was prepared by dissolving 24 g of PDB powder in 1000 ml distilled water and sterilized with autoclave at 121°C and pressure 15 for 15 minutes. After sterilization, the flask was left to cool. Before the medium hardened, the antibiotic Amoxicillin was added to 100 ml / l to prevent bacterial growth, then pour the medium into a petri dish.

3.2.4.Molecular diagnosis

3.2.4.1. *Trichoderma spp*

Trichoderma DNA was extracted from single spore cultures containing pure, young and actively growing hyphae using a modified Chelex®100 rapid extraction method (Ding et al., 2007). A chelex®100 Molecular Biology Grade Resin (BioRad Laboratories, USA) solution was made in sterile water and placed on a stirring plate to keep the resin suspended. For each sample, a small amount of mycelia from the edge of the *Trichoderma* colony was collected using the tip of a sterile 100 µL pipette tip. The mycelia was transferred into a 0.6 mL tube and 50 µL of 5% Chelex®100 solution added. The tubes were then placed on a heat block set at 95°C. After 10 min, the samples were removed and transferred immediately to a freezer at -20°C for 30 min. The samples were then rapidly thawed at room temperature and vortexed for 5 s before being centrifuged at 10000 g for 2 min. The aqueous layer which contained the DNA was carefully removed from the samples and placed into 0.2 mL tubes. The samples containing the extracted DNA were immediately stored in a freezer at -20°C. PCR Amplification :

The primer pair ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). (Integrated DNA Technologies company, USA) (White et al., 1990) using for amplification of ITS gene.PCR amplification was performed in a total volume of 25µl. mixture contained 5 µL of master mix, primers 1µL of the forward, and 1µL of the reverse, 16 µL of double-distilled H2O and 2 µL of genomic DNA,the reaction was performed in applied biosystem thermal cycler (Figure 3.2) denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 1min, 58°C for 1 min and 72 °C for 1 min, with final extension at 72 °C for 7 min. PCR products were separated on 1.5 % agarose gels containing ethidium bromide and visualized under UV light.

3.2.4.2.*M. phaseolina*

After *M. phaseolina* growne on PDA and incubated in dark at 25±1°C for 7 days, DNA of *M. phaseolina* was Extracted.CTAB protocol was used (Permingeat, 1998).

1. The advanced mycelium in the pet is taken with the help of spatula and put into the crushing bags.
2. 6002X CTAB buffer is added to these sachets and the mass of mycelium is crushed with a homogenizer from the outside of the sachet.
3. The homogenate is removed by pipette and placed in 1.5 ml eppendorf tubes.
4. 2 µl of mercaptoethanol (2-mercaptoethanol) is added into each tube and vortex and tubes are mixed.
5. Tubes are incubated in a 65 ° C water bath for 30-40 minutes.
6. After incubation, 600 µl chloroform-isoamyl alcohol (24: 1) is added to this homogenate and vortexed for 30-45 seconds.
7. Tubes are centrifuged at 14000 rpm for 10 minutes. New eppendorf tubes are overwritten while waiting for this process.
8. At the end of the centrifuge, two phases appear in the tube, the clear phase above is pipetted without contact to the substrate (200-250 µl) and placed in new tubes.
9. 200-250 µl of chloroform-isoamyl alcohol (24: 1) is added, vortexed and the liquid slightly clouded.
10. Tubes are centrifuged at 14000 rpm for 10 minutes. At the end of the centrifuge, two phases appear in the tube, the clear phase above is pipetted (about 200 µl) without touching the substrate and placed in new tubes.
11. 150 µl of cold isopropanol (2-propanol) is added to this liquid and the tubes are shaken gently 4-5 times. At this stage, DNA will bind to alcohol

and collapse to the bottom. Tubes are stored at -20 ° C for 20 minutes to reinforce precipitation.

12. The tubes are removed and centrifuged at 14000 rpm for 15 minutes, at which time the DNA collapses into the bottom of the tubes.
13. The contents of the tube are trashed and you can see the DNA at the bottom with a slight blur. If there is no residue at the bottom of the tube, this does not mean failure. Visible DNA fragments are present there. Also, a cream colored layer to be formed at the bottom of the tube may not be a pure DNA precipitate. At the centrifugation stage, the trash remained there. This DNA should now be washed with 70% alcohol. 1 ml of 70% alcohol is slowly added to the tube and poured immediately without shaking. It is necessary to drain the alcohol at the bottom of the tube, you can do this by inverting the tubes in paper towels in a sterile cabinet. If the alcohol is still there, it can be removed by tapping on the bottom of the tube but caution should be taken. Take care not to lose the pellet.
14. The DNA pellet was added to the 50 µl TE buffer for 10 minutes after drying in the sterile cabinet. This pellet is dissolved by small strokes until it is thoroughly blurred. The tubes are stored at -20 ° C.
15.

PCR Amplification :

The primer pair ITS5 (5'-TCCGTAGGTGAACCTGCG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3').(Integrated DNA Technologies company, USA) (White et al., 1990) using for amplification of ITSgene.PCR amplification was performed in a total volume of 25µl. mixture contained 2.5 µL of 10x buffer , primers 0.5 µL of the forward, and 0.5 µL of the reverse ; 19.5µL of double-distilled H2O,1 µL of dNTPs ; and 1 µL of genomic DNA,the reaction was performed in applied biosystem thermal cycler (Fig 3.2) denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 1min, 50°C for 1 min and 72 °C for 2 min, with final extension at 72 °C for 10 min. PCR

products were separated on 0.8% agarose gels containing ethidium bromide and visualized under UV light.

Figure 3.2. Applied Biosystems Thermal cycler PCR

3.2.5. DNA Sequencing

These sequences were submitted to the BLAST query tool (Altschul et al., 1997) and database (www.ncbi.nih.gov/blast/) for analysis and identification of ***M.phaseolina*** and ***Trichoderma spp*** phylotypes. Sequence alignments were performed manually. Finally, we selected 3 to 5 sequences for each phylotype, and phylogenetic analysis was carried out by distance analysis using the neighbor-joining method in MEGA7 and a gamma shape parameter of 0.1. Sequences selected from public databases belonging to known ***M.phaseolina*** and ***Trichoderma spp*** were included in the phylogenetic analysis.

The PCR products were used as templates for targeted DNA sequencing. Sequencing of both strands of the PCR product was performed on an ABI373 sequencing instrument according to the protocol supplied by the manufacturer using the Big Dye™Terminator Cycle Sequencing Ready Reaction Kit (PE

Applied Biosystems, Foster City, CA, USA). A cycle sequencing reaction was set up using the purified PCR product as below

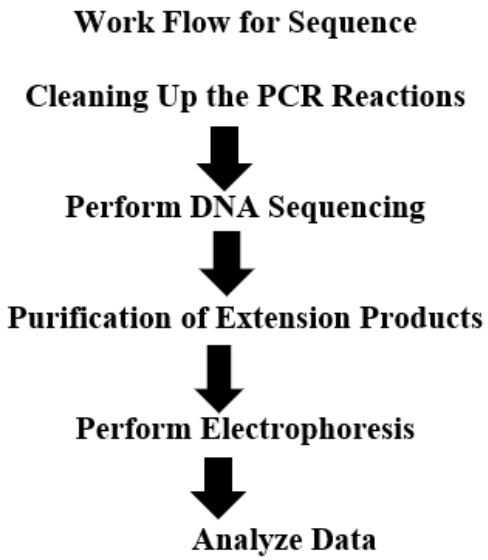

Figure 3.3.An overview of DNA sequencing procedures.

Table 3.2**.** Primers used to amplify and sequence

	Primer	**Sequence (5′→3′)**	**Amplicn size (bp)**
M.phaseolina	ITS5	TCCGTAGGTGAACCTGCG	**700**
Trichode ma spp	ITS4 GC	TCC TCC GCT TAT TGA TAT	**550**

To Clean Up the PCR Products :

1. 2 µl of GML ExoSAP was added to each 5 µl amplified reaction and the plate with MicroAmp Clear Adhesive Cover Film was sealed.

2. Vortex the plate briefly to mix the contents and Centrifuge the plate at 1600 g for 30 seconds.
3. Set the reaction volume to 7 µl and program the thermal cycler as shown in table (3.5)

Table 3.3. Program the thermal cycler conditions for Clean Up the PCR Products

Stage	Description	Temp.	Time
1	Enzyme Incubation	37 oC	15 min
2	Enzyme Inactivation	80 oC	15 min
3	Hold	4 oC	Indefinite Hold

To Perform DNA Sequencing

Preparing the Sequencing Reactions for Perform DNA Sequencing as follows :

Table 3.4. Reaction mixture for the perform DNA sequnencing reactions

Component	**Volume µL per specimen**
BigDye Terminator Mix	1.0 µL
Sequencing Buffer	2.0 µL
Sequencing Forward Primer	2.0 µL
Distilled Water	3.0 µL
Total	8.0 µL

Program the thermal cycler conditions for perform DNA sequencing stage as follows:

96°C	1 min	
96°C	10 sec	40 cycles
50°C	5 sec	
60°C	4 min	
4 oC	∞	

To Purification of Extension Products

1. 700 µL Sephadex mix was added (1 g Sephadex + 14 mL distilled water) into a 2 ml tube.

   Note: Before using Sephadex mix please vortex and shake it gently minimum 30 minutes.
2. The tube was centrifuged for 2 minutes at 4500 rpm and collecting tube with flow-through was discarded.
3. The column was placed into a new 2 ml collecting tube or into the same back and whole PCR product was added to the center of column.
4. The tube was centrifuged for 2 minutes at 4500 rpm and PCR product was at the bottom of the tube and ready to load on instrument.

Figure 3.4. Applied Biosystems 3130xl Genetic Analyzer

3.2.6. Preparation Filtres of *Trichoderma spp*

For testing the antifungal activity of *Trichoderma* isolates, three mycelial agar plugs randomly were taken from the PDA colony of a 4 day old, plugs were inoculated inoculated in 100 ml potato dextrose broth (PDB) in a 250-ml Erlenmeyer flask, in shake culture (150 rpm) at room tempercture for 15 days. Cultural filtrates were obtained by centrifuging the cultures at 12,000 rpm and

filtering through 0.22 μm membrane filters to remove hyphal fragments and were stored at 4°C until use.

3.2.7. Antifungal assay

The PDA and SDA mediums by 400 ml were Prepared and divide the medium into 4 glass flasks 100 ml for each medium, where 5 ,10 and 15 ml of *Trichderma spp* filtrate were placed for the flasks, then pouring the culture medium into the dishes after that the dishes were pollinated with pathogenic fungus *M.phaseolina* and *F. oxysporum*, into PDA. For *T. tonsurans* and *M.canis* into SDA were incubated at 28° C and the results were taken after the pathogenic mushrooms covered the entire area of the dish in the comparison treatment

3.2.8. Dual cultural screening of antagonistic fungi

Antifungal activity of each *Trichoderma* isolate against *M.phaseolina*, *F.oxysporum* were tested in dual cultures on PDA. Mycelial agar plug of Trichoderma (6 mm in diameter) were placed on a PDA dish (9 cm in diameter) at one centimeter from the edge. A mycelial agar plug (6 mm in diameter) of pathogens were placed 7 cm from the Trichoderma, inoculant to establish a dual culture. There were five dishes (replicates) for each isolate. The cultures were incubated at 28°C for 7 days. For *T. tonsurans* and *M.canis* fungal were tested in dual cultures on SDA. As Trichophyton grows a much slower than Trichoderma it's homogenate was spread over two-thirds of SDA 9 cm Petri dish and incubated for 2 days at 28°C. Trichoderma hypae disc was then placed over the empty third of the Petri dish. Antagonism was identified by overgrowth of *Trichoderma spp*. on *Trichophyton spp*. within 7 days from inoculation. *Trichoderma spp*. overgrowth was quantified as the distance of overgrowth (cm) (El-Naggar, Kövics, Karaffa, & Irinyi, 2008).

$$R = \frac{A - B}{A} \times 100$$

where: R – percentage reduction in the growth of pathogen.

A – radius (cm) of pathogen colony in control culture.

B – radius (cm) of pathogen colony in test dish.

3.2.9.Statistical analysis

The experiment was carried out according to the design (C.R.D) under in vitro condition and data were analyzed for variance (ANOVA) using the of SAS software version (9.1.) The mean was compared with the least significant difference (L.S.D) at a level of 0.05 .

4. RESULT AND DISCUSSION

4.1. Isolation of *Trichoderma spp*.

A total of 4 fungal isolates were successfully isolated from different samples. Preliminary screening was carried out which showed that these fungal isolates were Trichoderma species. The types of samples and the isolate codes were shown in Table 4.1.

Table 4.1. Isolate codes and the types of sample used.

Isolate Code	Types of Sample
A	Soil
S	Soil
H	Compost
ASH	Compost

4.1.1. Morphological identification

Colony characters of Trichoderma isolates were studied using 7 days old PDA cultures that were incubated at 28°C. Trichoderma isolates grew well 4 days. Morphological identification of the potential Trichoderma isolates was performed by (Anees et al., 2010), based on the colony appearance and pigmentation, the sizes of conidia, the branching patterns of conidiophores and the presence or absence of chlamydospores.

The conidial production in **A** was diffused, dispersed . The colour of mature conidia was grayish green. For **S**, the mature conidia appeared to be light green, and concentric ring was observed. The conidia tend to concentrate at the center of the colony. For **H** isolate dark green conidia tend to form in pustules, More concentric rings were observed in these colonies compared to anther colonies.

In the colonies of **ASH,** Their conidial productions were restricted to the center of the colonies, diffused, and appeared to be yellowish green,and arranged in concentric rings. (Fig.4.1).

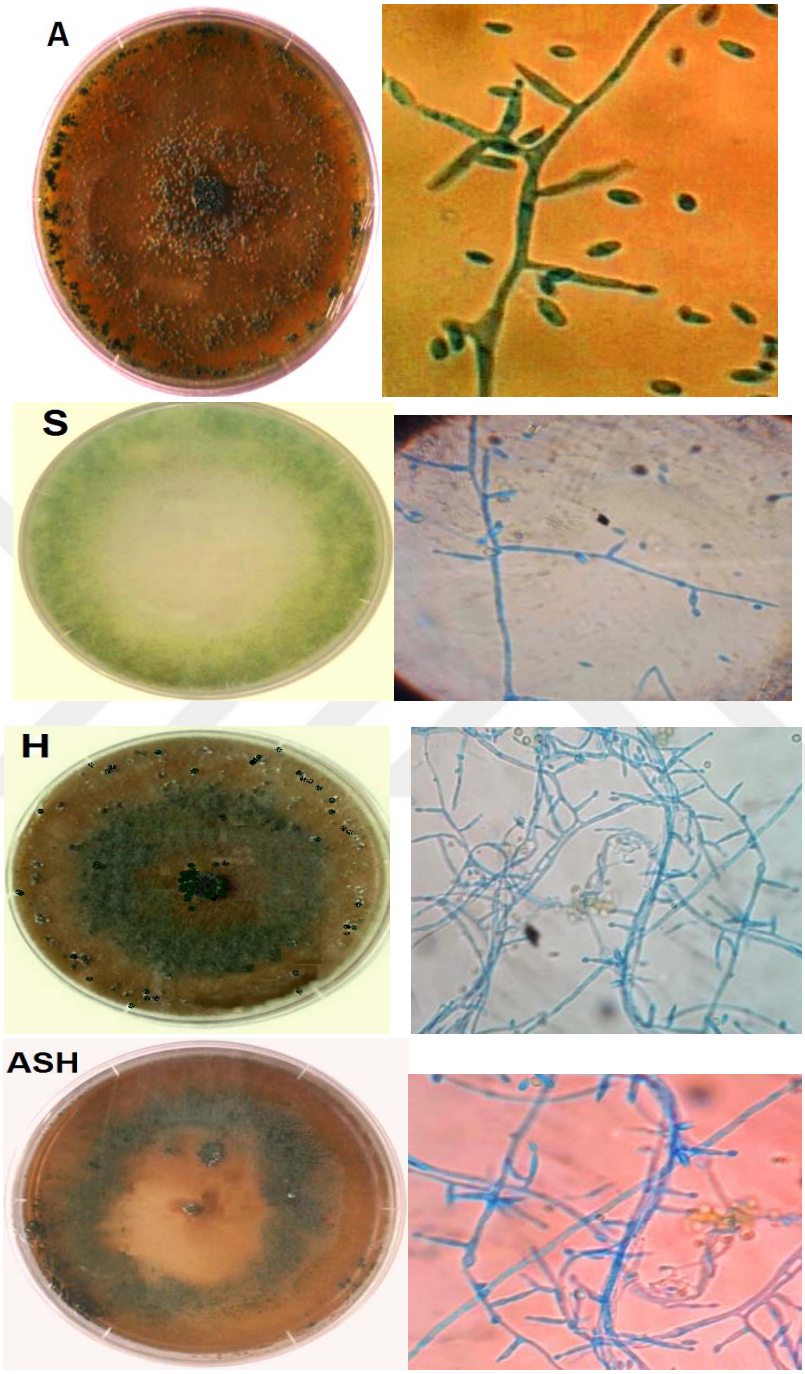

Figure 4.1. *Trichoderma* isolates on PDA at 28±2°C after 7 days of incubation, (Left) Colony color, (Right) Microscopic features (40X)

However, information from morphological study alone is insufficient to precisely identify a Trichoderma species because *Trichoderma* species have relatively few morphological characters and limited variation that may cause overlapping and misidentification of the isolates (Anees et al., 2010). Besides that, morphological characteristics are influenced by culture conditions (Diguta, Vincent, Guilloux-Benatier, Alexandre, & Rousseaux, 2011). Therefore, there is a necessity to use molecular technique to compensate for the limitations of morphological characterization.

4.1.2. Molecular identification of *Trichoderma* isolates

The ITS1/ITS4, as well as ITS regions for Trichoderma isolates were amplified, and approximately the product sizes of 500 bp (Figure 4.2).

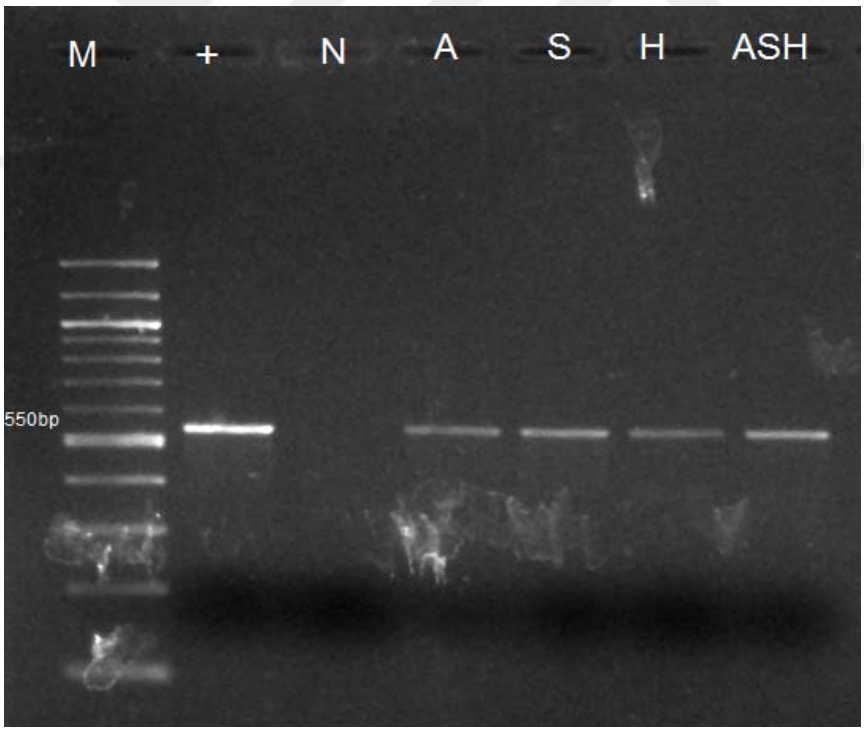

Figure 4.2. 550 bpITS region amplified for all *Trichoderma* isolates using primer pair ITS1 and ITS4. M: 100 bp DNA ladder.+: Positve. N: Negetive A: *T. viride*.S: *T. Virens*.H: *T. Harzianum*. ASH: *T. lixii*.

4.1.3 Sequence Analysis of *Trichoderma* isolates

PCR products amplified from all *Trichoderma* isolates were sequenced. They could be aligned and a consensus sequence was generated from each alignment made . Then, BLAST program were used to determine the species identity of *Trichoderma* isolates. DNA sequencing of the ITS region is one of the most reliable loci for the identification of a strain at the species level (Kullnig-Gradinger et al., 2002). By comparing the sequences of the ITS region to the sequences deposited in GenBank, The BLAST search results were presented in (Fig4.3),all of the *Trichoderma* isolates was identified to species level with homology percentage of at least 96%. The results obtained from the morphological interactive key, BLAST search tools were found to be in agreement. the four isolates, *T. viride* (A), *T. Virens* (S), *T. Harzianum* (H) and *T. lixii* (ASH), were identified

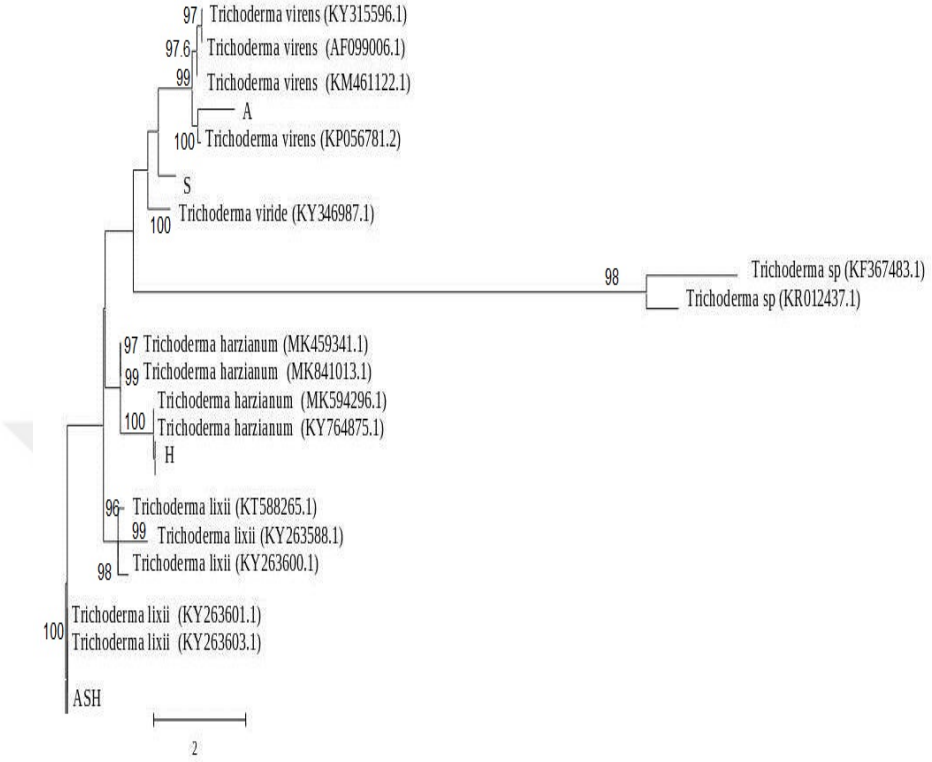

Figure 4.3. Phylogenetic tree based on internal transcribed spacer (ITS) sequences of the selected *Trichoderma* isolates. The tree was generated from the ITS sequences of *Trichoderma* isolates by the neighbor-joining method using MEGA10 (http://www/ megasoftware.net/).

The BLAST search of NCBI followed by phylogenetic sequence analysis of *Trichderma* isolates, A had 97-100% sequence identity with *Trichoderma* virens with the accession number in a GenBank (KY315596.1, AF099006.1, KM461122.1, KP056781.2). S had 100% sequence identity with T*richoderma vride* with the accession number in a GenBank (KY346987.1) and 98% with *Trichoderma sp* with the accession number in a GenBank (KF367483.1, MN102106.1). H had 97-100% sequence identity with *Trichoderma harzianum* with the accession number in a GenBank (MK459341.1, MK841013.1, MK594296.1, KY764875.1). ASH had 96-100% sequence identity with

Trichoderma lixii with the accession number in a GenBank (KY263601.1, KY263603.1, KY263600.1, KY263588.1, KT588265.1).

4.2. Molecular diagnosis of *M.phaseolina*

An approximately 550 to 700 bp of ITS DNA fragment was successfully amplified from all *M.phaseolina* (Fig.4.4)

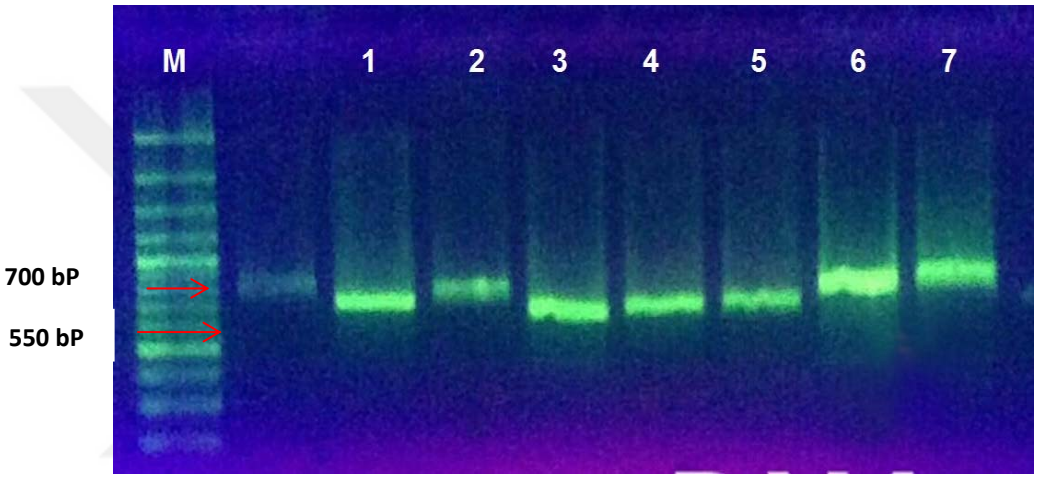

Figure 4.4**.** PCR detection of *M.phaseolina* by (ITS4-ITS5) primers

4.2.1.Sequence Analysis of *M.phaseolina*

Morphological and molecular approaches were shown to play important roles in the identification of *M.phaseolina* isolates. Each approach has its own limitations and strengths. By combining morphological and molecular approaches, all *M.phaseolina* isolates were successfully identified. The results obtained from the BLAST search tools were found to be Primer ITS4 and ITS5, were used to amplify by PCR to the DNA of *M.phaseolina*, Compared with the identity of rDNA sequence of M. phaseolina from NCBI GenBank databases by blast method, th e result of the sequencing showed that was found and its homology was up to 100 %(accession number: KP191626.1) Fig 4.5

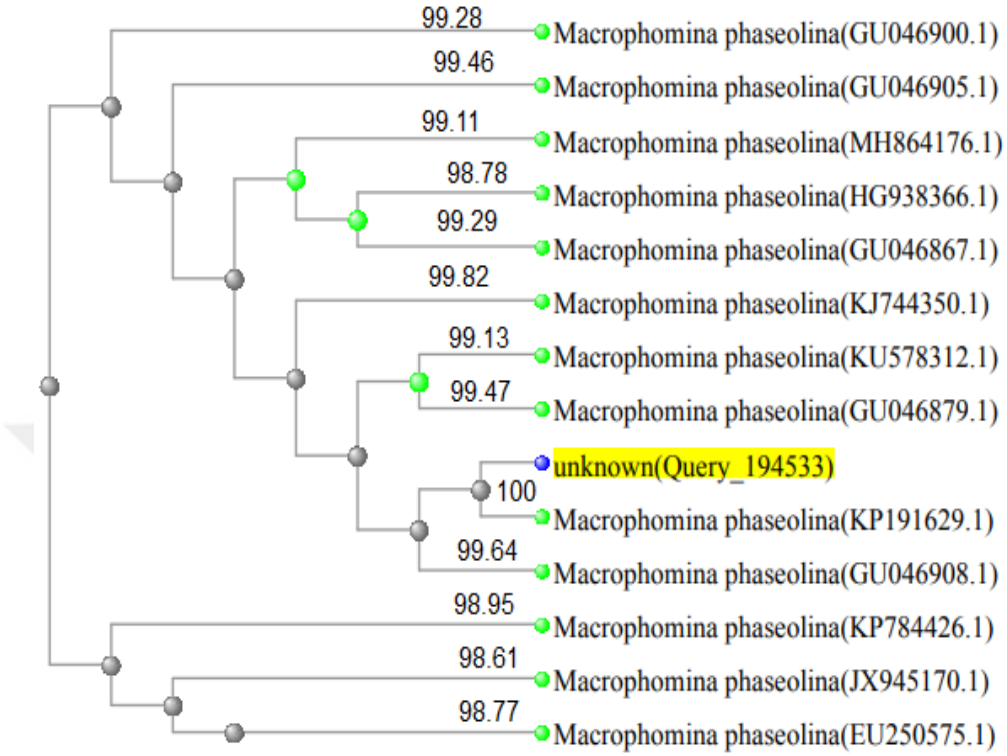

Figure 4.5|. Neighbour-joining tree showing representatives of sequence type identified in this work (in yellow), and reference sequences from Genbank (in black).

4.3. Molecular diagnosis of *T.tonsurans, M canis* and *F. oxyporum*

TheITS regions for both *T.tonsurans, M canis* and *F. Oxysporum*. *T.tonsurans, M canis*, were amplified and approximately the product sizes of 600 bp (Fig 4.5). For *F. oxysporum*,500 bp sizes were obtained (Fig4.6).

Molecular identification of the positive cultures of dermatophytes was achieved for digestion the PCR products which resulted from amplification of ITS region in rDNA gene. In the ITS region was amplified by using the primers ITS1 and ITS4 which resulted in PCR products ranged between 600- 630 bp. These results showed good similarity with the results of (Ghojoghi et al., 2015)

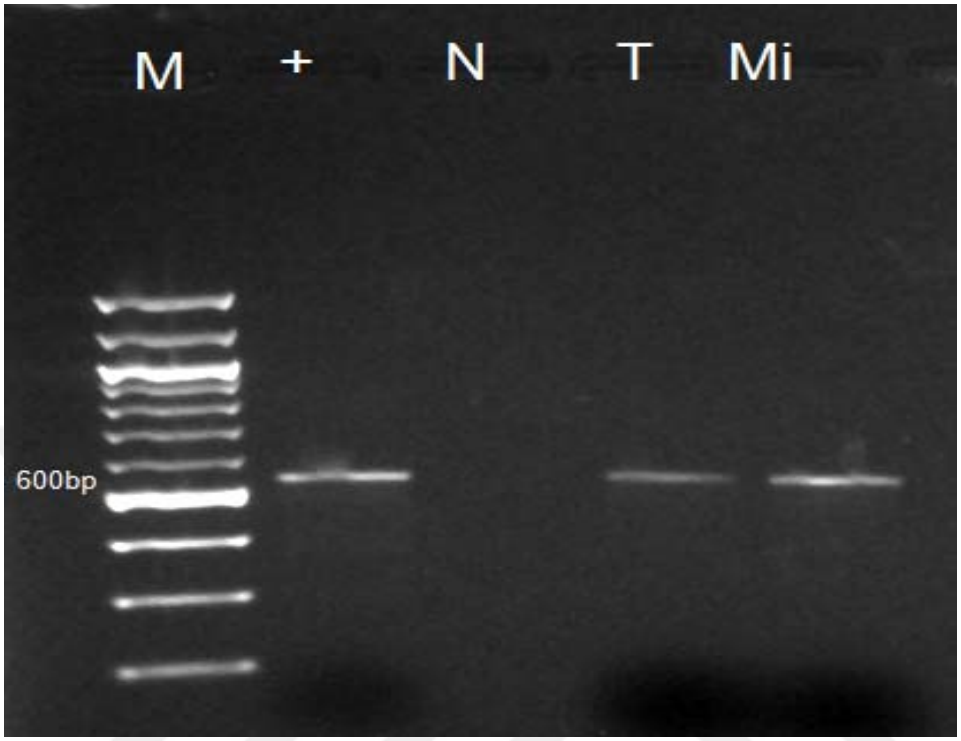

Figure 4.6**:** 600 bp ITS region amplified for T:*T.tonsurans* and Mi: *M canis*. .+: Positve. N: Negetive, using primer pair ITS1 and ITS4. M: 150 bp.

With the aim of checking the identify of the F.oxysporum, DNA performed was Amplified fragments of almost 500 bp (Fig4.6), obtained by PCR and was compared with reasultes (Singha, Kakoty, Unni, Das, & Kalita, 2016) .

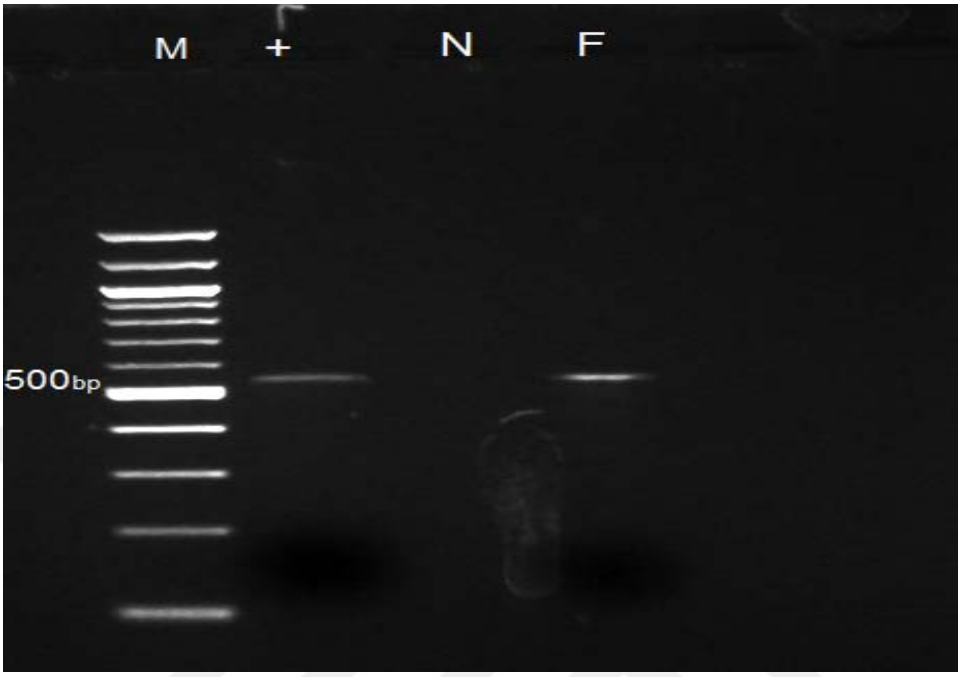

Figure 4.7**:** 500 bp ITS region amplified for F: *F. oxyporum*, .+: Positve. N: Negetive. using primer pair ITS1 and ITS4. M: 100 bp.

4.4 Effect of filtrates *Trichoderma* isolates in inhibition of *F.oxyporum* and *M.phaseolina*.

Data in Table (4.1) indicated that, All studied control treatmens superior on inhibition zone parameter of *F.oxyporum* and *M.phaseolina* compared with the pathogens alone, the highest value was in *T. harzianum* treatment of the pathogenic fungus in all of the concentrations, the inhibition in which reached 4.66, 2.66, 0.00, 4.33, 2.00 and 0.00 cm respectively, compared to 9.00 cm, in present of the pathogens alone. This inhibition might be due to antibiosis, nutrient competition or cell wall degrading enzymes, This finding is in agreement with (S. Kumar, 2013) .As for the concentrations they all led to increased in inhibition, but concentrate 15 m/l caused maximum inhibition of the pathogens in *T. harzianum* treatment, in which reached 0.00 and 0.00 cm respectively, compared to 9.00 cm in present of the pathogens alone. The results obtained suggested that *Trichoderma spp*. is

capable of producing a range of antibaiotic, which have antifungal activity. *T. viride, T. virens,T. harzianum* and *T. lixii*. significantly (P≤0.05) inhibited the growth of fungal pathogens, indicating antagonistic properties against the pathogens. Somasekhara, Anilkumar, and Siddarad (1996). The *Trichoderma spp* used in this study were found agreement with Nikam, Jagtap, and Sontakke (2007), who reported that *T. harzianum* was more effective in inhibiting growth of fungal pathogens. Production of chitinolytic and glucanolytic enzymes from *T. harzianum* may have direct significance in the parasitism on pathogens as these enzymes function by breaking down the polysaccharides, chitin and β-glucan that are responsible for the rigidity of fungal cell walls and interact synergistically to achieve a high level of antifungal activity, thereby destroying cell wall integrity, which could explain the strong antagonistic results of *T. harzianum* (Howell, 2003).

Table 4.2. Effect of filtrates *Trichoderma* isolates in inhibition of *F.oxyporum* and *M.phaseolina*.

	Inhibition Zone (cm)					
	F.oxyporum			***M.phaseolina***		
Isolates	5 ml/L	10 ml/L	15 ml/L	5 ml/L	10 ml/L	15 ml/L
T. viride	5.00	4.00	1.00	500	3.00	1.33
T. virens	5.67	3.66	1.33	5.33	3.33	0.66
T. harzianum	4.66	2.66	0.00	4.33	2.00	0.00
T. lixii	5.33	4.33	0.33	5.00	2.66	0.33
Control	9.00	9.00	9.00	9.00	9.00	9.00
L.S.D P<0.05	0.81	1.40	1.05	1.32	1.33	1.15

4.5. Effect of filtrates Trichoderma isolates on percentage of inhibition of *F.oxyporum* and *M.phaseolina*

The obtained data, table 4.2. revealed that all *Trichoderma* isolates had a considerable antagonistic effect on mycelial growth of *M. phaseolina* and *F.oxysporum*, when compared to the control (the pathogens alone). *T.harzianum*

showed the best rate of inhibiting especially at the concentrations 15 ml/L, in which the ratio of inhibition reached 100%, and this results agreemen with Ghisalberti and Sivasithamparam (1991).

Table 4.3. Effect of filtrates *Trichoderma* isolates on percentage of inhibition of *F.oxyporum* and *M.phaseolina*.

	Inhibition index (%)					
	F. oxyporum			***M.phaseolina***		
Isolates	5 ml/L	10 ml/L	15 ml/L	5 ml/L	10 ml/L	15 ml/L
T. viride	44.40	55.56	88.90	44.53	66.67	85.23
T. virens	37.06	59.30	85.20	40.80	62.96	92.60
T. harzianum	48.16	70.40	100.00	51.76	77.80	100.00
T. lixii	40.76	51.90	96.30	44.43	70.46	96.47
Control	0.00	0.00	0.00	0.00	0.00	0.00
L.S.D P<0.05	9.00	15.66	11.65	14.66	14.79	12.75

4.6.Effect of filtrates *Trichoderma* isolates on the growth inhibition of *T.tonsurans* and *M. canis.*

Results showed in table 4.3. that all the *Trichoderma* isolates achieved a high antifungal activity against *T. tonsurans* and *M. canis.* The growth inhibition of pathogenic fungi increased with increasing of filtrates concentrations.the highest value was in *T. harzianum* treatment in 15 m/l concentration, the inhibition in which reached 0.50 and 0.46 cm respectively, compared to the control (pathogens alone) 9.00 cm. Might the extract contains active pharmacological compound which could be responsible for the rate of inhibition. This results in agreement with Al-Obaidy and Al-Rijabo (2011).

Table 4.4. Effect of filtrates *Trichoderma* isolates on the growth inhibition of *T.tonsurans* and *M. canis.*

Inhibition Zone (cm)

	T.tonsurans			***M canis***		
Isolates	5 ml/L	10 ml/L	15 ml/L	5 ml/L	10 ml/L	15 ml/L
T. viride	4.67	3.86	1.03	5.40	3.33	0.86
T. virens	4.17	4.10	0.83	4.80	3.40	0.80
T. harzianum	4.33	3.6	0.50	4.56	3.13	0.46
T. lixii	4.73	3.93	0.90	5.06	3.90	0.93
Control	8.00	8.00	8.00	8.00	8.00	8.00
L.S.D P<0.05	0.73	0.97	0.66	0.84	0.68	0.40

4.7.Effect of filtrates *Trichoderma* isolates on percentage of inhibition of *T.tonsurans* and *M canis*

Results showed that all isolates of *Trichoderma* lead to increased percentage inhibited mycelial growth of *T.tonsurans* and *M canis* . *T. harzianum* isolate showed deffrent significant inhibition of growth in 15 ml concentration, in which reached 93.75 and 94.17 % respectively. (Table 4.4).. The rate of inhibition might be due to creating anti-microbial agents extracellular lytic proteins and lytic enzymes. In this study, Trichoderma spp were resulted as effective antagonists inhibiting the growth of pathogenic dermatophyte fungi, the results being similar to those shown by Omero et al. (2004).

Table 4.5. Effect of filtrates *Trichoderma* isolates on percentage of inhibition of *T.tonsurans* and *M canis*

Inhibition index (%)

	T.tonsurans			***M canis***		
Isolates	5 ml/L	10 ml/L	15 ml/L	5 ml/L	10 ml/L	15 ml/L
T. viride	41.68	54.17	87.08	32.50	58.33	89.17
T. virens	47.91	45.25	89.58	42.91	57.50	90.00
T. harzianum	45.83	48.16	93.75	40.00	60.83	94.17
T. lixii	37.98	50.83	88.75	36.66	48.75	88.33
Control	0.00	0.00	0.00	0.00	0.00	0.00
L.S.D P<0.05	9.19	12.87	8.30	10.55	8.49	5.09

4.8. Effect of different *Trichoderma* isolates for inhibition of *M.phaseoilna* and *F.oxyporum* in Dual culture.

Antagonistic effects of all *Trichoderma* isolates were tested against *F. oxysporum* and *M.phaseolina* on PDA at 28°C for 7 days. The reasults in table 4.5. indicates that there were significant differences between *Trichoderma* isolates used to inhibit of *F. oxysporum* and *M.phaseolina*. Among all *Trichoderma* isolates, (*T.lixii*) exhibited the lowest inhibition to the growth of *F. Oxysporum* (2.66 cm). whereas (*T. harzianum*) showed the highest inhibition percentage (1.33cm) . In the antagonistic test against *M.phaseolina*,(*T.viride*) exhibited the lowest antagonistic capacity inhibition of (2.86cm) while (*T. harzianum*) exhibited the highest percentage of inhibition, (1.66cm).

The obtained data revealed that all *Trichderma* isolates used in this study had a considerable antagonistic effect on mycelial growth of F.oxysporum and M. phaseolina in dual cultures, when compared to the controls. Out of all the tested Trichoderma isolates, the isolates *T. harzianum* showed a maximum inhibition against growth of the pathogen, in which reached 1.33 and 1.66 cm respectively.Numerous reports indicated that *Trichoderma spp*. isolates (Haque & Ghaffar, 1992) ,are effective biocontrol agents, and these indications support our results. The in vitro screening of five isolates of *T. harzianum*, one isolate of *Chaetomium globosum*, and one isolate of *Conetherium mentance*, revealed that all of them significantly reduced the growth area of *M. phaseolina, Fusarium solani*, and *Rhizoctonia solani* (Hermosa et al., 2000). The higher antagonistic effect of T. *harzianum* against *M. phaseolina* compared to *T. viride* was previously observed by. Sreedevi et al. (2011). This finding is in agreement with our results on the potent antagonistic activity of *T. harzianum* isolate against F.*oxysporum* and *M. phaseolina*. This antagonistic nature might be due to antibiosis, nutrient competition, and cell wall degrading enzymes (S. Kumar, 2013).

Table 4.6. Average inhibition of *F. oxysporum* and *M.phaseolina* by different *Trichoderma* isolates in Dual culture.

	Inhibition Zone (cm)	
isolates	***F. oxysporum***	***M.phaseolina***
T. viride	2.33	2.86
T. virens	2.66	1.86
T. harzianum	1.33	1.66
T. lixii	2.66	2.42
Control	9.00	9. 00
L.S.D P<0.05	0.93	0.69

4.9.Effect of different *Trichoderma* isolates in the percentage for inhibition of *F.oxyporum* and *M.phaseoilna*

The comparison of the data obtained from the dual culture revealed that all 4 isolates of *Trichoderma spp* inhibited the growth of *F. oxysporum* and *M. phaseolina* ranged from 68.16 to 81.50 % and 70.43 to 85.20 respectively, (Fig 4.6).The results indicated that *T. harzianum* isolate suppressed the pathogens growth and caused maximum inhibitionof the growth of *F.oxysporum* and *M. phaseolin*a was reached to 81.50 and 85.20%,respectively. The rate of inhibition might be due to producting volatile secondary metabolites like as hydrogen cyanide, ketones , ethylene and aldehydes, which play an important role in controlling various pathogens.This study in agreement with the one conducted by (Vey, Hoagland, & Butt, 2001). Similar to this finding,Reino, Guerrero, Hernández-Galán, and Collado (2008), reported that potential of *Trichoderma spp*. to produce many volatile (e.g. pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g. terpenes).

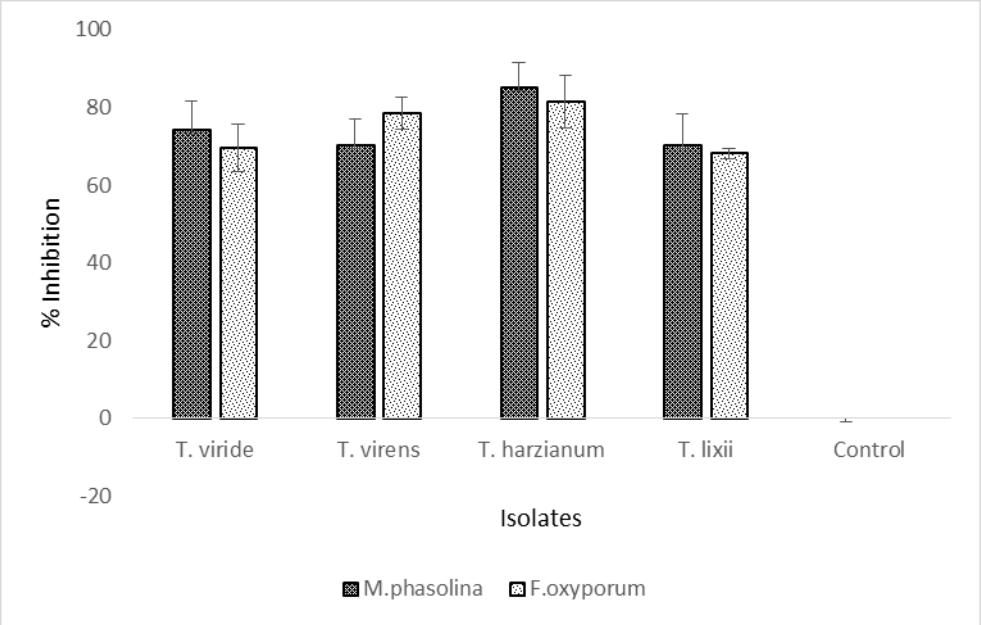

Figure 4.8. Effect of different *Trichoderma* isolates in the percentage for inhibition of *F.oxyporum* and *M.phaseoilna.*

4.10. Average inhibition percentage of *T.tonsurans* and *M canis* by different *Trichoderma* isolates in Dual caltuer .

Reasults indicated in Table 4.1. All *Trichoderma* isolates superior on inhibition of *T.tonsurans* and *M canis* compared with the pathogens alone, the highest value was in *T. harzianum* of the pathogenic fungus with both pathogen, the inhibition in which reached 1.43 and 1.50 cm respectively, compared to 7. 50 cm, in present of the pathogens alone. whereas *T. viride* showed the lowest inhibition to the growth of *T.tonsurans* an inhibition 2.33 cm. while *T. Virens* showed the lowest inhibition to the growth of *M canis* an inhibition 2.20 cm. The dual culture of *Trichoderma spp* and *dermatophytes* in culture media led to a variety of interactions. dermatophytes growth was generally inhibited, similar reactions have been reported on other fungal pathogens (Hanson & Howell, 2004). Mycoparasitism behaviour displayed by Trichoderma spp. was evidenced by the

microscopic observations of the interaction regions between *dermatophytes* and *T. viride,T. virens,T. harzianum* and *T. lixii.* The mycelia of *Trichoderma* isolates grew on the surface of the pathogen always coil round their mycelia and later penetrate their cell walls directly without formation of appressorium structures. The mycelia of the pathogen then disintegrate suggesting an enzyme action (Omero et al., 2004; Padmodaya & Reddy, 1996). Metcalf and Wilson (2001) demonstrated the possible role of chitinolytic and glucanases enzymes in the biocontrol exhibited by *Trichoderma.* These enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity limiting the growth of the pathogen.

Table 4.7. Average inhibition percentage of *T.tonsurans* and *M canis* by different *Trichoderma* isolates in Dual caltuer .

	Inhibition Zone (cm)	
isolates	***T.tonsurans***	***M canis***
T. viride	2.33	1.66
T. virens	2.00	2.33
T. harzianum	1.34	1.50
T. lixii	1.50	2.00
Control	7.50	7. 50
L.S.D P<0.05	1.12	1.09

4.11.Effect of different *Trichoderma* isolates in the percentage for inhibition of *T.tonsurans* and *M canis*

All of the tested *Trichoderma* isolates restricted the growth of *T.tonsurans* and *M canis* colonies Fig 4.7. The average level of this growth inhibition varied between 68.86-82.30 %, respectively with both pathogens. *T. harzianum* isolates showed a high level of antagonistic activity in which reached 82.30 and 80.10 %, respectively. In comparison with the other *Trichoderma* isolates.

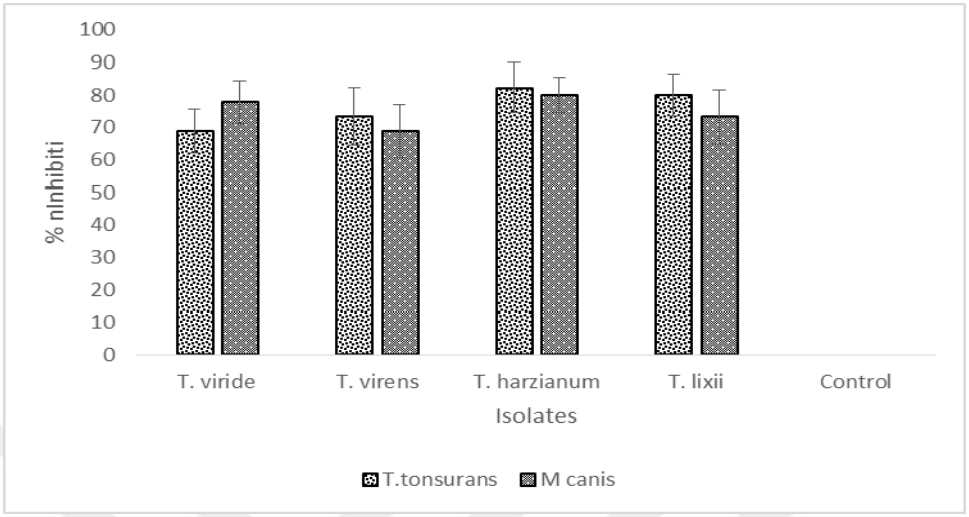

Figure 4.9. Effect of different *Trichoderma* isolates in the percentage for inhibition of *T.tonsurans* and *M canis*

5. CONCLUSION

In this study, Molecular identification the *Trichoderma spp* using ITS and the effect of different concentrations filtrates of *Trichoderma spp* on the growth of the plant and human pathogens, were investigated.. Moreover, isolates were brought from Adana and included in PCR study. The optimum concentration for the growth inhibition of pathogens were 15ml/L.

Four isolates of *Trichderma spp* were subjected to the antagonistic test, and all isolates were able to increas inhibition of fungi pathogens under vitro conditions. As a result, *T.harzianum* isolate, showed promising results in inhibition of both plant and human pathogens. Based on these results, the following recommendations were listed:

- Current study confirmed that under the conducive environmental condition, plant (M.phaseolina and F.oxysporum) and human (T.tonsurans and M. canis) pathogens can cause a serious problems. Therefore, controlling it using chemical fungal treatments may not be advisable, It may also develop resistance against treatments and to be are unsafe to the human health. Therefore, the use of alternative disease management strategies like use of *Trichoderma spp* single or with other bio-control agents.
- From the antagonistic assay results, it was noticed that *T. harzianum* isolate were more effective in all concentrations than the others in reducing growth of pathogens as (*M.phaseolina* , *F.oxysporum*, *T.tonsurans* and *M. canis*). Therefore, it is advisable to screen the efficacy of bio-agents of the different species under vitro conditions before use.

- Up to recently, approximately more than 90% of Trichoderma species have been isolated. However, the current study pointed out that it is possible to isolate Trichoderma spp easily from soil and organic composts. Therefore, future studies should pay attention to several substrates, mainly, forest soil, different organic compost sources (Animal, plant and natural) where several biodiversities may available. They should also be tested against this fungus.

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