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Biological Control of Tomato Early Blight by Using Native Isolates of *Trichoderma* spp.

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List of Abbreviation

Item	Abb.	Item	Abb.
Trichoderma harzianum	*T. harzianum*	Potato dextrose broth	PDB
Trichoderma	*T.*	Potato dextrose agar	PDA
Trichoderma Selective Media	TSM	Wettable powder	W.P
Hour	Hr	Pathogenesis related	PR
Alternaria solani	*A. solani*	Sterile distilled water	SDW
Gram	Gm	Control	CK
Milliter	Ml	Active ingredient	a.i
Distilled Water	DW	Biocontrol agent	BCA
Liter	L	Relative humidity	RH
Colony Forming Unit	CFU	spp.	Species
Part per million	*ppm*	Manbegan®	M
Centimeter	Cm	Antracol®	A
Round per minute	Rpm	Daconile®	D
Microgram	µg	Manzidan®	M
Milligram	Mg	Rovral ®	R
Pathogenesis related	PR	polyethylene	PE
Systemically acquired resistance	SAR	Induced systemtic resistance	ISR
cell-wall-degrading enzymes	CWDs	Salicylic acid	SA

ABSTRACT

Early blight, caused by the fungus *Alternaria solani*, is one of the most common diseases of tomato, potato, pepper and eggplant. The fungus attacks leaves, stems and fruit. Tomato early blight can be controlled by cultural practices, fungicides, and biologically by using the bioagent *Trichoderma*. The fungus *Trichoderma* is a very common saprophytic fungus. Seventy three isolates of *Trichoderma* species were recovered from soil samples collected from different locations in the West Bank. The antagonistic potential of the obtained isolates and others against early blight disease was investigated in bioassays under growth chamber conditions. Results showed that the isolates (Jn14, Jn58, Jn17 and T34) reduced disease by 70%, 58%, 52% and 40%, respectively. *Trichoderma* isolates reached a peak in mycelium growth rate at 25°C and growth was completely inhibited at 35°C. The mode of action of *Trichoderma harzianum* includes antibiosis; the mycelia growth rate of *A. solani* was significantly reduced due to the production of fungitoxic substances by the *Trichoderma* isolates in liquid culture at 25°C. *T. harzianum* isolate (Jn14) was the most effective isolate against the pathogen; it reduced *A. solani* mycelium growth rate by (72%) at the temperature of 25°C and at the concentration of 25% (v/v).

Concerning formulation, the *T. harzianum* isolates (Jn14 and Jn58) were applied as spore suspension and talc formulation. The decrease in disease was significant in both methods at the concentrations of $10^7$and 10^8 CFU/ml on tomato plants; disease was reduced by (58% and 54%) as talc and (48% and 45%) as spore suspension, respectively.

Five fungicides, (Propineb, Iprodione, Chlorothalonil, Maneb and

Mancozeb) were found to be effective in reducing mycelium growth rate and spore's germination of *A. solani* at the concentrations of (0.5, 1, 3, 5 and 7µg ml^{-1}) in comparison with the control. The fungicides Iprodione, Chlorothalonil, Propineb, Mancozeb and Maneb at the concentration (3µg ml^{-1}), significantly reduced disease severity by 76%, 56%, 52%, 44% and 40%, respectively, compared to the control.

Concerning integrated control, the combination of *T. harzianum* (Jn14) and the fungicides (Iprodione and Propineb) at the concentration of 3µg ml^{-1} reduced early blight disease on plants in pots by 40% and 15% respectively, compared to the control. In field application, the combination of *T.harzianum* (Jn14) at the concentration of 10^8 CFU/ml and the fungicides (Iprodione, Propineb) at the concentration of (3µg ml^{-1}) reduced early blight disease severity in the field by 31% and 25% respectively, compared to the control. Application of *Trichoderma* with fungicides was better than using *Trichoderma* alone. The combination of *T. harzianum* with fungicides improved the level of disease control; fungicides however, were obviously better in respect to disease reduction.

Chapter One

1. General Introduction

1.1 Early blight

1.1.1 Importance

Early blight of tomato, caused by *Alternaria solani* (Rotem, 1994) is considered economically as the most important disease of tomatoes in many areas of the world, where significant reductions in yield (35 to 78 %) have been observed (Jones *et al.*, 1993). The disease, which in severe cases can lead to complete defoliation, is the most damaging on tomato (Peralta *et al*., 2005) in regions with heavy rainfall, high humidity and fairly high temperatures (24-29°C).

The most important hosts of *A. solani* are tomato, potato and eggplant (Pscheidt, 1985). Other hosts included horse nettle, chilli, black nightshade and non-solanaceous hosts such as wild cabbage, cucumber and zinnia (Neergaard, 1945). The disease is less frequent and less damaging on pepper and eggplants. Weakened plant tissues, either due to stress, senescence, or wounding, are more susceptible to *Alternaria* infection than healthy tissues (Thomma, 2003).

1.1.2 Causal Agent

A. solani belongs to the Fungi Imperfecti (Deuteromycotina) in the class Hyphomycetes and order Hyphales (Agrios, 2005). An Ascomycete fungus, *Pleospora solani*, has been claimed (Esquivel, 1984) as the teleomorphic stage of *A. solani*, but this has not been confirmed by others. *A.solani* belongs to the large-spored group within the genus *Alternaria* which is characterized by separate conidia borne singly on simple conidiophores (Neergaard, 1945). The conidia of *A. solani* are muriform and beaked (Neergaard, 1945).

Like other members of the genus *Alternaria*, *A. solani* has transverse

and longitudinal septate conidia, multinucleate cells, and dark-colored (melanized) cells (Rotem, 1994). Melanin gives protection against adverse environmental conditions including resistance to antagonistic microbes and their hydrolytic enzymes (Rotem, 1994). *Alternaria* spp. has no known sexual stage or over wintering spores, but the fungus can survive as mycelium or spores on decaying plant debris for a considerable period of time, or as a latent infection in seeds (Rotem, 1994).

1.1. 3 Damage

As discussed by Rotem (1994), yield is reduced by the destruction of foliage and the fruits are damaged directly by the pathogen and by sun blotch on defoliated plants. The disease progressively weakens the plant and increases its susceptibility to infection by reducing the photosynthetic leaf area and increasing the imbalance between nutrient demand in the fruits and nutrient supply from the leaves (Rotem, 1994). *Alternaria* has the ability to grow over a wide range of temperatures from 4 to 36 °C (Vloutoglou and Kalogerakis, 2000) and requires only a short wet period of at least four hours for successful infection (Vloutoglou and Kalogerakis, 2000).

The two major features of *Alternaria* spp. are the production of melanin, especially in the spores, and the production of non-specific as well as host-specific toxins in the case of pathogenic species (Thomma, 2003). One of the earliest identified nonspecific toxins is alternaric acid, identified by (Thomma, 2003). This toxin, isolated from lesions or from culture filtrate, caused chlorosis and necrosis when introduced in tomato plants and also damaged nonhosts of *Alternaria* like cabbage, radish, spinach, pea, bean, and others, thus pointing to its non specificity (Langsdorf *et al.*, 1990).

Under favorable conditions, *Alternaria* spores germinate within hours and can produce more than one germ tube per spore as the spores consist of several cells. The ability to penetrate the cuticule, stomata, and wounds has

been described for most *Alternaria* spp. (Rotem, 1994). In the case of less virulent strains or young and therefore resistant leaves, the germinating hyphae of *A. cassiae* and *A. alternata* tend to spread over the intact leaf surface and the only sites of infection are dead cells, suggesting that penetration is preconditioned by the secretion of toxins (Van Dyke and Trigiano, 1987).

1.1.4 Symptoms

The first symptoms of the disease are small, dark and necrotic lesions that usually appear on the older leaves and spread upward as the plants become older (Sherf and Macnab, 1986). As lesions enlarge, concentric rings are obvious giving a target board-like appearance and are often surrounded by a yellow zone. In severe epidemics *A. solani* can cause premature defoliation, which weakens the plants and exposes the fruit to injury from sunscald (Sherf and Macnab, 1986).

As explained by Sherf and Macnab (1986), large, dark and sunken lesions may appear on the stems of seedlings at the ground line, causing partial girdling known as collar rot. Seedlings are weakened and die when the stem is completely girdled by the lesion. On the main stem and side branches of adult plants, the fungus causes small, dark, slightly sunken areas that enlarge to form dark brown elongated spots, which occasionally show concentric rings like those on the leaves. These spots are scattered along the stem and branches (Gardner, 1990).

On green or ripe fruits, dark, velvety, sunken spots may occur at the stem end. These spots occasionally develop from mycelia extension from stem lesions, reach a considerable size and may show distinct concentric markings like those on the leaves (Sherf and Macnab, 1986). Semi-ripe fruits are more susceptible than ripe ones (Mehta *et al.*, 1975). Heavily infected fruits frequently drop before they mature. On susceptible genotypes, the

calyx and blossom may also become infected (Pandey *et al.*, 2003).

1.1.5 Epidemiology

The primary inoculum produces conidia in the spring, which are then splash or wind dispersed to the lower leaves of the plant, where they germinate and infect (Rotem, 1994). Wind, rain and insects are the primary methods of dissemination of the pathogen (Rotem, 1994). *A. solani* has typical dry-dispersed spores; the conidia have slightly rough surfaces with dark-colored walls and are produced away from the host surface on aerial conidiophores (Fitt *et al.*, 1989). Such dry-dispersed spores may be removed from the host by gusts of wind, by acceleration forces as leaves moves by the wind, and by rain or overhead irrigation splash, through puff or tap mechanisms (Fitt and Walklate, 1989). Pscheidt and Stevenson (1988) have shown that on potato plants, sporulation occurs at temperatures between 5 and 30°C, with the optimum around 20°C. *A. solani* causes early blight on potato and tomato wherever the host is grown, and is most severe in warmer than cooler environments (Rotem, 1998). Inside host tissues, conidiophores form at night or during the day in low temperatures (Honda and Nemoto, 1984); sporulation increases as chlorotic tissues become nectrotic (Bashi and Rotem, 1975b), and requires a long wet period or interrupted short wettings (Rotem, 1998) caused by rain, dewfall, or high relative humidity.

The minimum, optimum and maximum temperatures for conidia productions are 10, 22.5, and 30°C, respectively. Conidiophores production has a greater range of possible temperatures, including 5, 27.5, and 35°C, (Bashi and Rotem, 1975a). Conidia detach in the wind as they are carried out to new hosts by wind, splashing rain, or insects. Susceptibility to infection is determined primarily by the age of the host plant; *A. solani* causes collar rot in tomato seedlings and early blight in mature tomato and potato (Rotem, 1998). The Colorado potato beetle and flea beetles facilitate the transmission

of early blight to tomato (Heuberger and Dimond, 1941). Foliage infection takes place when germ tubes penetrate the epidermis directly or through the stomata.

The minimum, optimum, and maximum temperatures for spore germination are reported as 4, 28, and 40 °C, respectively (Waggoner and Parlange, 1975). Long wetting periods or free moisture availability also encourages germination. Infection is accompanied by the production of toxins by *A. solani*, including some non-host specific toxins called alternaric acid, zinniol, altersolanol, and macrosporin. These toxins cause disease in the host plant by acting on the plant protoplast to disturb physiological processes that sustain plant health (Agrios, 1997). Photosynthesis increases and respiration decreases partially throughout the production of enzymes and toxins on hosts (Rotem, 1998). *Alternaria solani* may survive in the soil for over 10 years in debris and seeds at optimum temperatures, or only a few years otherwise (Rotem, 1998).

1.1.6 Disease Control

1.1.6.1 Cultural methods

1.1.6.1.1 Rotation and intercropping

The benefits of crop rotations on soil health and disease management are well known in agricultural systems. However, as most vegetable growers have specialized in fewer crops in order to remain competitive, intensive production systems have led to the adoption of short-term crop rotations with low biodiversity (Hill and Ngouajio, 2005). Westcott (2001) recommends planning a 3-year rotation to control *A. solani* in potato or tomato. Removing and burning of infected debris and eradication of weed hosts further reduces inoculum (Agrios, 1997).

Hanafi (2003) found that rotation is hardly used in greenhouse production systems and in most greenhouses, continuous cropping is

practiced, without or with very short fallow (crop-free) interval. It may be desirable to cultivate more than one variety of tomato during a growing season, since varieties differ in terms of susceptibility to early blight.

Smith and Kotcon (2002) found that intercropping by using a resistant tomato cultivar such as 'Juliet' with the susceptible cultivar such as 'Brandywine' reduced the rate of disease increase of early blight in the susceptible 'Brandywine' compared to a 'Brandywine' monoculture. However, the presence of resistant foliage reduced disease spread during the season. In addition, they found the same results for other intercrop experiments between resistant and susceptible vegetables (such as lettuce and tomato) for disease reduction.

1.1.6.1.2 Resistant cultivars

Cultivars with good levels of field resistance are available. However, no immunity to early blight has been found in commercial tomato cultivars or in their wild parents. Field resistance to foliage infection is associated with plant maturity. Thus, late maturing cultivars are usually more resistant than early maturing cultivars and therefore, one should avoid planting early and late cultivars in the same or adjacent fields (Shoemaker and Gardner, 1986).

Resistant cultivars are potentially the most economical control measure as they can extend the fungicide spray intervals while maintaining control of the disease (Shtienberg *et al.,* 1995).

Breeding for disease resistance requires efficient screening techniques, genetic resources for resistance, knowledge of genetic and physiological mechanisms underlying resistance, and appropriate breeding strategies to transfer resistance genes into improved genetic backgrounds. Compared with many other diseases of tomato, limited progress has been made to improve early blight resistance of tomato cultivars.

Major difficulties in the past breeding efforts have been with the

screening of plants for resistance and transferring of resistance genes across genotypes (Martin and Hepperly, 1987). Early blight is associated with the physiological maturity of the plant; older leaves are more susceptible than younger leaves, and a heavy fruit set enhances the disease (Maiero and Barksdale, 1989). Furthermore, plants with late maturity, indeterminate growth habit, or low yielding ability appear to be more resistant (Maiero and Barksdale, 1989.). In the same concern, resistance has been characterized as horizontal, controlled by polygenes, and highly affected by environmental conditions (Nash and Gardner, 1988). Various techniques have been employed for screening tomato plants for early blight resistance. Field screening has been a routine procedure (Shoemaker and Gardner, 1986).

In organic crop production, where synthetic fungicides are not allowed, growers often rely on resistance cultivars as a means for disease control. In addition, organic growers in the U.S. often grow rare 'heirloom' varieties for their unique appearance or taste; however resistance to *A. solani* is not well characterized in these varieties (Rotem, 2000). Cultivars that are listed as 'resistant' in catalogs, such as 'Juliet' and 'Mountain Supreme', may still show symptoms of early blight, but lesion expansion is slower, or spore production may be inhibited. In addition, many of the more rare varieties listed in catalogs have not been screened for resistance (Maiero *et al*., 1991). In 1942, the U.S. Breeding Laboratory, in Charleston, South Carolina, tested hundreds of wild tomato species for resistance to *A. solani* (Rotem, 2000). The basis for commercial cultivar resistance to *A. solani* comes mainly from crossing *Lycopersican esculentum* with *L. pimpinellifolium, L. hirsutum*, and *L. peruvianum.* Unfortunately, resistance in the stem has a higher coefficient of heritabiltiy than resistance in the leaves (Stancheva, 1991).

1.1.6.1.3 Compost

Composts incorporated into soil or planting mixes can provide effective biological control of diseases caused by soil borne plant pathogens (Hardy and Sivasithamparam, 1991). They also may reduce the severity of diseases caused by foliar plant pathogens (Miller *et al*., 1997). Composts may improve the ability of plants to resist diseases caused by root as well as foliar pathogens by inducing systemic resistance in plants (Han *et al*., 2000, Zhang *et al*., 1996). The components of composts responsible for this induced activity may be biological or chemical in nature (Zhang *et al*., 1996). Roe *et al*. (1993) reported lower incidence of early blight of tomato on field-grown plants in compost amended soil than in the control, even though the compost was applied under polyethylene mulch.

Systemic acquired resistance (SAR) can be induced by chemicals, pathogens, and beneficial soil microorganisms (De Meyer and Höfte, 1997). It is unknown how composts induce SAR. However, plants in compost-amended substrates are colonized by a variety of bacterial taxa (Workneh and Bruggen, 1994) from which strains capable of inducing systemic resistance in plants have been described (Liu *et al.*, 1995). Such specific strains must be present above a certain threshold population size in the rhizosphere to induce this effect (Raaijmakers *et al.*, 1995).

Compost water extracts have been used for years as topical sprays to control foliar diseases of plants (Yohalem *et al*, 1994). They reduced the severity of foliar diseases, such as grey mold of strawberries, and late blight of potato (Elad and Shtienberg, 1994). These treatments often were variable in efficacy. However, the effect of the substrate (soil) in which the plant was grown may have an impact on the efficacy of compost water extracts, but this potential interaction had not been investigated.

Compost extracts contained biocontrol agents, as well as unidentified chemical factors that appear to play a role in efficacy (Cronin *et al.*, 1996).

Although the mechanisms by which these extracts provide control remained largely a mystery, Cronin *et al.* (1996) demonstrated that a low molecular weight compound was critical for *in vitro* lysis of conidia of *Venturia inaequalis*. It has been postulated that the protective effects of compost extracts is due, at least in part, to the induction of systemic resistance in plants. Chemicals, such as salicylic acid (SA), can induce SAR in plants (De Meyer and Höfte, 1997). Comparison of the disease control achieved by compost water extracts with a putative chemical inducer for SAR, such as SA, may aid in understanding the mechanisms of disease suppression induced by these extracts.

1.1.6.1.4 Fertilization

Host nutrition is an important factor affecting the severity of *Alternaria* epidemics (Goodman *et al.,* 1986). Rotem (1981) first noticed that low nitrogen levels were associated with greater disease severity on tomato plants; high nitrogen levels resulted in less disease, but also reduced yield. This observation was confirmed and elaborated upon subsequently by numerous studies (Barclay and Hutchinson, 1973). It was found that low levels of nitrogen and potssium increased *Alternaria* severity, whereas high levels were associated with mild disease severity on potatoes (Barclay and Hutchinson, 1973; MacKenzie, 1981) and tomatoes (Rotem, 1981).

High nitrogen levels are known to prolong plant vigor and delay maturity, especially when other factors are limiting. Since *Alternaria* is known to be primarily a pathogen of senescing tissue (Rotem, 1994), any factor which delays maturity will also reduce the severity of the disease. Over-fertilizing tomato plants with nitrogen has been shown to produce luxurious, green plants which are virtually immune to early blight (Hillocks and Chindoya, 1989). This is thought to be due to the effects of nitrogen and fruit load on physiological age of the plant tissue. High fruit load and low

nitrogen both hasten senescence and therefore increase the susceptibility of the plants to blight. Barclay *et al.*, (1973) suggested that at high nitrogen levels the period of meristematic activity in the plants is extended, allowing the plant to restrict the spread of infection.

The possibility of controlling *Alternaria* diseases by surplus application of nitrogen was examined in several studies and various trials met with some success (MacKenzie, 1981). However, in practice, control of *Alternaria* by adding more nitrogen to the soil is economically not advantageous. There is a great difference between the fertilizer rate for optimal disease suppression and the rate for optimal yield. Other drawbacks to high levels of fertilizer were the hazard to groundwater and, in potatoes, a reduction in tuber quality. Therefore, MacKenzie (1981) concluded that the crop should be fertilized for optimum yield and *Alternaria* should be managed by properly timed applications of fungicides during the growing season.

1.1.6.2 Physical methods

1.1.6.2.1 Elimination of certain wavelength in greenhouse

Alternaria, like many of plant pathogenic fungi sporulates only when they receive light in the ultraviolet range (below 360 nm). It has been possible to control disease on greenhouse vegetables caused by several fungi species by covering or constructing the greenhouse with a special UV-absorbing vinyl film that blocks transmission of light wavelengths below 390nm (Agrios, 1997).

As light was involved in the pathogenic processes of several foliar diseases, light transmitted through the greenhouse covers was considered as the sole controllable factor in the spread of foliar diseases in unheated and passively ventilated low-technology walk-in tunnels and greenhouses, where relative humidity (RH) was frequently very close to 100% at night, as

reviewed recently (Raviv and Reuveni, 1995). UV- B radiation (280 to 320 nm) induced sporulation in *B. cinerea* and other pathogenic fungi, while monochromatic blue light inhibited this process (Furuya, 1986). Tan and Epton (1973) clearly demonstrated that sporulation in some *B. cinerea* isolates, which occurs in the dark, can be inhibited by blue light. In fungi, light acts in many cases as a stress-inducing agent and results in morphogenetic changes, including spore production and germination. These findings suggested that careful modification of the light spectrum might efficiently reduce spore production and germination. According to Honda *et al*., (1968), the control of light quality can be used to reduce the inoculum potential of *B. cinerea* in the greenhouse. Partial control of gray mold on cucumber and tomato had been reported by Honda *et al*. (1968), who used vinyl films that absorb UV light shorter than 390 nm.

Sasaki *et al*. (1985) claimed that the part of the UV spectrum active in reduction of sporulation was 300 to 340 nm. Control of early blight of greenhouse tomato caused by *A. solani* had also been achieved by inhibition of sporulation by means of UV-absorbing vinyl film (Vakalounakis, 1991). However, Jordan and Hunter (1972) found higher, rather than lower, levels of *Botrytis* infection on strawberries grown under several colored polyethylene (PE) films compared with those grown under clear PE films or glass. In this case, the blue (PE) caused two synergistic phenomena related to *Botrytis* infection: etiolated plants and decreased vapor pressure deficit and temperature. Illumination with blue light had been reported to inhibit sporangial production of *Psedoperonospora cubensis* in infected cucumber leaves (Jordan and Hunter, 1972), but no practical use of this finding was suggested.

In a previous study, spectrally modified (PE) sheets with various ratios of blue to UV-B transmission were investigated for their effects on the epidemiology of gray mold caused by the fungus *B. cinerea*. The results

showed a remarkable reduction in sporulation of *B. cinerea* (Reuveni *et al.*, 1989) as well as a slower rate of gray mold epidemic development on tomatoes in greenhouses covered with (PE) sheet having a high blue/UV transmittance ratio (Reuveni and Raviv, 1992). The pigment used in this sheet caused light absorption with a peak at 580 nm.

1.1.6.3 Chemical methods

Fungicide treatments are generally the most effective control measures, but are not economically feasible in all areas of the world and may not be effective under weather conditions favorable for epidemics (Herriot *et al*., 1986). However, fungicides are commonly used to control early blight and consist of Protestants products like mancozeb (Dithane M-45) and chlorothalonil (Bravo), or systemic fungicides belonging to the strobilurin class (Bartlett *et al.,* 2002). Strobilurin compounds are site specific fungicides, and although very effective initially, resistance has been identified for a number of fungi including early blight (Bartlett *et al*., 2002; Pasche *et al.,* 2004).

Fungicide treatments are the most effective way to control early blight to a non-damaging level (Manohara, 1977). Typically, fungicides are applied starting from two weeks after transplanting until two weeks before harvest at two- to three- week intervals, but in the wet season a fungicide treatment once or twice per week is necessary (Manohara, 1977). Such heavy use of chemicals is not economically feasible for the generally resources-limited growers. It also imposes health concerns for growers and consumers as well as environmental hazards. In the long run the intensive use of fungicides could stimulate the emergence of resistant variants of the fungus in many countries, in a similar way as has been reported recently in the U.S. (Pasche *et al.*, 2004).

In the absence of fungicide treatment, maximum fruit infection for

susceptible varieties was about 30%. Potential yield processing tomato and fruit size are reduced on an average of 30% and 10%, respectively (Sherf and Macnab, 1986). Sinha and Prasad (1991) tested seven fungicides in the field over 3 seasons against *A. solani*. Dithane M-45 (Mancozeb) ® 0.2% was the best and effective treatment with the highest yield.

Khade and Joi (1980) reported that nine fungicides were able to reduce disease incidence, but the highest yield increases were obtained with Dithane M-45 (Mancozeb), blue Cu 50, Cuman L, Dithane Z-78 (Zineb) and Difolatan (Captafol). Choulwar and Datar (1989) reported that in tests, sprays of 0.2% Mancozeb applied at different times after transplanting significantly reduced the intensity of early blight caused by *A. solani*. Vidhyasekaran (1983) reported that both Mancozeb and Captafol effectively controlled *A. solani* and *Septoria lycopersici*. These treatments reduced defoliation and increased fruit production. Fruits from sprayed plots had significantly more sugars and vitamins and less phenolics.

1.1.6.4 Biological methods

Biological control is a non chemical measure that has been reported in several cases to be as effective as chemical control (Elad and Zimand, 1993). However, the efficacy of biological control was occasionally inadequate and variability in control level may be high. Understanding the mechanisms involved in biological control might enable enhancing control efficacy and reducing the inconsistency and variability. The mechanisms involved in biological control were several and included, among others, induced resistance, competition for nutrients, and secretion of inhibitory compounds.

Biocontrol products can serve as an alternative to some chemical fungicides, especially in case of fungicide failure, if they offer acceptable levels of disease control. Only a few micro-organisms have been fully commercialized for the control of foliar plant pathogens (Fravel *et al.,* 1999).

Several pathogens, biotrophs and necrotrophs of cucumber were controlled by *T. harzianum* (T39) under commercial conditions.This biocontrol agent (BCA) controled the foliar pathogens, *Botrytis cinerea*, *Pseuperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* (syn. *S. fuliginea*) in cucumber under commercial greenhouse (Elad, 2000 a, b; Elad and Shtienberg, 1994). As mentioned above T39 also controlled powdery mildew and downy mildew; these findings confirmed previous results with powdery mildew, obtained in smaller scale experiments (Elad *et al.,* 1998a), and represent the first detailed report of downy mildew control by (T39). The suppression of these two diseases as a result of *Trichoderma* spray was unexpected, as T39 had been developed for the control of necrotrophs such as *B. cinerea* and *S. sclerotiorum*, and very few examples of powdery mildew control by *Trichoderma* spp. had been reported (Elad *et al.*, 1998a). The biocontrol agent was as good as the standard chemical treatment.

1.1.6.4.1 Induced resistance

An alternative to usual chemical plant protection agents could be the use of induced resistance. **Two kinds of induced resistance**

(1) The systemically acquired resistance or (SAR).

(2) The rhizobacteria - mediated induced systemic resistance, or (ISR).

SAR is activated after infection by a necrotising pathogen or other biotic and abiotic stresses; uninfected plant parts acquire resistance then towards a broad spectrum of pathogens. SAR, effective against many types of pathogens including viruses, is associated with the production of pathogenesis related (PR) proteins and is mediated via a salicylic acid (SA) dependent process (Heller and Gessler, 1986). After an acquisition period that ranges from few hours to several days, this effect lasts for weeks and also protects the growing plant.

The systemic signal prepares the tissue to react more rapidly and more

efficiently to an infection challenge by a virulent pathogen. This phenomenon is often referred to as "conditioning" or "sensitizing" (Sticher *et al.*, 1997). Since SAR protects the plants against a variety of diseases, it is assumed that the use of general chemical plant protection agents could be substantially reduced if substituted by SAR. It is likely that a combination of the resistance inducer with a fungicide in low dosage would give best results. This fungicide addition would stop the further development of attacking pathogens during the resistance build-up when the plants are still susceptible.

SAR can also be induced by a variety of chemicals which do not act systemically but cause lesion-like tissue damage at the points of application, suggesting that these chemicals mimic the biological SAR induction by necrotizing pathogens. Examples for these chemicals are various salts, unsaturated fatty acids, harpin proteins, elicitin peptides, and sublethal concentrations of certain herbicides, as reviewed by (Oostendorp *et al.*, 2001). All these compounds do not have direct antimicrobial effects on the pathogens.

Rhizobacteria, a saprophytic bacterium that live in the plant rhizosphere and colonize the root system, have been studied as plant growth promoters for increasing agricultural production and as biocontrol agents against plant diseases (Burris, 1998). The use of rhizobacteria as inducers of systemic resistance (ISR) is an alternative tool for disease control. There are many reports in the literature on the use of rhizobacteria as biocontrol agents in diverse pathosystems, under field conditions (Wei *et al.*, 1996). Rhizobacteria induced resistance works against a broad spectrum of pathogens, including bacteria, fungi, nematodes and viruses (Maurhofer *et al.*, 1994) and their use represents an advantage over classical biological and chemical control methods, in which plants are typically protected against only a few pathogens. Different rhizobacteria utilize different mechanisms for triggering systemic resistance; some trigger the SA-dependent pathway,

others jasmonate/ethylene dependent one, and additional pathways are likely to be discovered in the future (Pieterse *et al.*, 2001).

1.2 *Trichoderma* species

1.2.1 Biology and Taxonomy

Trichoderma are among the most common saprophytic fungi. They are within the subdivision Deuteromycotina which represents the fungi lacking or having an unknown sexual state (though many *Trichoderma* are considered asexual). Further, it is part of the form class hyphomycetes. They are known as early invaders of roots and quickly occupy an ecological niche on the roots. Due to their ability to utilize substrates they do not completely depend on the plant in their life cycle. They are also considered cellulolytic ascomycetes and among the organisms responsible for the destruction of cellulosic fabrics (Elsas and Wellington, 1997).

Rifai (1969) distinguished nine species differentiated primarily by conidiophore branching patterns and conidium morphology based on microscopic characters, *including T. harzianum, T. viride, T. koningii, T. hamatum, T. longibrachiatum, T. pilulifrum, T. polyporum, T.aureoviride and T. pseudokoningii* .

A sectional classification was proposed for *Trichoderma* by recognizing the following sections; section *Trichoderma, Longibrachiatum, Saturnisporum, Pachybasium and Hypocreeanum* (Bissett, 1991a). Twenty species were assigned to *Trichoderma* section *Pachybasium.* They were described and differentiated on the basis of conidiophore and conidium morphology (Bissett, 1991b). In the section *Trichoderma*, characterized species by narrow and flexuous conidiophores with branches and phialides uncrowded, frequently paired, and seldom in verticals of more than three.

In the section *longibrachiatum,* Bissett (1984) indicated that conidiophores are sparingly and irregularly branched, with irregularly disposed and not usually in whorls or verticals and species in this section produce

distinctive greenish yellow pigments in reveres of cultures. In the section *Saturnisporum,* conidiophores have a branching system with branches and phialides uncrowned and frequently paired and compact conidiogenous pustules as in section *Pachybasium.* However, it was differentiated by the bullate or winglike conidial ornamentation. The section *Pachybasium*, had species with highly ramified, broad conidiophores usually arranged in compact pustules or fascicles, and with branches and phialides broad or inflated, relatively short, and disposed in crowded verticals. Some species are characterized by the production of sterile conidiophores. The section *Hypocreeanum*, distinguished by effuse, usually spars conidiation, sparingly branched conidiophores, and cylindrical to subulate phialides (Bissett, 1991a).

1.2.2 Ecology

Trichoderma spp. are widely distributed all over the world (Domsch *et al.,* 1980) and occur in nearly all soils and other natural habitats, especially in those containing organic matter (Papavizas *et al.,* 1984). *Trichoderma* spp. seems to be a secondary colonizer, as its frequent isolation from well-decomposed organic matter (Danielson and Davey, 1973). *Trichoderma* spp. is also found on root surfaces of various plants (Davet, 1979); on decaying bark, especially when it is damaged by other fungi; and on sclerotia or other propagules of other fungi (Davet, 1979).

The abundance of *Trichoderma* spp. in various soils coupled with their ability to degrade various organic substrates in soil, their metabolic versatility, and their resistance to microbial inhibitors, suggests that they may possess the ability to survive in many ecological niches depending on prevailing conditions and the species or strain involved. Some of the conidia of *T. harzianum* added to soil without nutrient– supplying amendments survived between 110 and 130 days, but the length of survival depended on the isolate used (Papaviza, 1981; Papaviza *et al*., 1982). Most of the conidia

probably lysed without first germination, or they germinated in response to some nutrients released from organic matter and subsequently lysed in the absence of food bases adequate enough to sustain further growth and sporulation. Conidia added to soil decreased initially, then stabilized for two years to about one – tenth the original number added (Davet, 1979). Hyphae also have the ability to survive in soil (Papavizas *et al*., 1984).

Lewis and Papavizas (1984) demonstrated the potential of various *Trichoderma* species aggregates to form chlamydospores readily and in great numbers in natural soil or in fragments of organic matter after the introduction of the fungus to the soil as conidia. They suggested that introduced isolates have the potential and aggressiveness to colonize and establish themselves in organic matter in natural environments.

Acidic pH levels enhance *in vitro* growth of *T. harzianum* and stimulate its chlamydospores formation and conidial germination (Chet and Baker, 1980)**.** In addition, soil moisture enhances the *Trichoderma* conidia to survive longer than in dry soil (Lui and Baker, 1980). *Trichoderma* can tolerate fungicides, such as methyl bromide, captan and maneb (Rupple *et al*., 1983).

Lewis and Papavizas (1984) demonstrated, however, that the number of colony forming units (CFU) of *T. harzianum* and *T. viride* introduced four days before, or at the time of fumigation with sublethal rate of sodium methane, was significantly less than those in non fumigated soil and the number of colony forming units (CFU) of strains introduced four days after fumigation was similar to that in non fumigated soil.

1.2.3 *Trichoderma,* The bioagent

Trichoderma species can attack and control plant pathogenic fungi, and have potential as biocontrol agents (Estrella and Chet, 1998). Hence, there is a growing interest in the physiology and molecular genetics of the

mycoparasitic interactions of *Trichoderma.* Parasitism by *Trichoderma* spp. is necrotrophic, resulting in the death of the host fungus.

There are little reports in the literature on the use of *Trichoderma* spp. as biocontrol agents of *A. solani. Trichoderma* spp. is commonly used in biological control of soilborne and foliar diseases (Elad *et al*., 1999).

Gullino and Garibaldi (1994) showed the reduction of tomato leaf blight disease caused by *A. solani* when *Trichoderma* spp. was included in the integrated management. Six species of *Trichoderma* including *T. harzianum, T. viride, T. koningii, T. hamatum, T. longibrachiatum and T. pseudokoningii* were tested. These antagonists were tested against the mycelia growth of the pathogen and disease incidence on tomato plants.

T. harzianum (T39), the active ingredient of the commercial preparation TRICHODEX, has been used for the control of foliar diseases such as grey mould in various crops, and of *Pseudoperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* in cucumber and *Cladosporium fulvum* in tomato (Elad *et al*., 1999; Elad, 2000 a, b) under commercial greenhouse conditions.

T. harzianum (T39) when applied alone was as effective as the standard chemical treatment in 70% of the trials. In (20%) of the experiments, disease suppression achieved by *T. harzianum* (T39) was significantly inferior to that of the standard chemical treatment. In the other (10%) experiments, *T. harzianum* (T39) efficacy was insufficient. However, Inconsistency and variability in control efficacy among biocontrol agents are common (Elad, 1990).

T. harzianum (T39) induces plant defense against *Botrytis cinerea* in tomato, lettuce, pepper, bean, and tobacco. Application of *T. harzianum* (T39) at a site spatially separate from inoculation with *B. cinerea* results in a 25 to 100% reduction in disease severity (De Meyer *et al*., 1998).

Integration of biological and chemical controls has been investigated in

some studies. Alternation of *T. harzianum* (T39) with chemical fungicides on a weekly basis resulted in disease suppression that was as effective as that achieved by applying the fungicide alone as being more consistent than *T. harzianum* (T39) applied alone (Elad and Zimand, 1993; O'Neill et al., 1996). Although the alternating treatment was effective and provided a 50% reduction in the number of chemical sprays, biocontrol agent was applied at pre determined and fixed intervals. *T. harzianum* (T39) is a living organism that is affected by the environment. Accordingly, it is possible that under certain conditions application of this agent would provide significant control but that under other conditions a fungicide would be more effective (Elad and Zimand, 1992).

Biocontrol of the necrotrophic fungus *Botrytis cinerea* by *Trichoderma* spp. can be mediated by mechanisms that either directly or indirectly affects *B. cinerea* development. Direct modes of action include mycoparasitism (Elad and Zimand, 1993) and production of inhibitory compounds (Tronsmo and Dennis, 1977). Examples of indirect mechanisms are competition for nutrients (Zimand *et al.*, 1995) and space (Dobos and Bulite, 1981) because the presence of *Trichoderma* spp. changes the environment for *B. cinerea* development. These different strategies are not mutually exclusive since *T. harzianum* (T39) combines competition for nutrients (Zimand *et al.,* 1995) and interference with pathogenicity enzymes to control *B. cinerea* (Zimand *et al.,* 1996).

Some studies have demonstrated that *Trichoderma* spp. can also affect the host plant (Ryals *et al.,* 1996). Addition of *T. viride* cellulase to grapevine cell cultures induced plant defence reactions such as the hypersensitive response and phytoalexin production (Calderon *et al.,* 1993). A similar induction of plant defence reactions by *T. longibrachiatum* in tobacco plants was linked to an increased resistance to *Phytophthora parasitica* var. *nicotianae* (Chang *et al.,* 1997). This suggests an indirect biocontrol effect of

Trichoderma spp. through the induction of plant resistance.

Krause *et al*. (2003) demonstrated that less than 2% of 80 different batches of composts tested induced systemic resistance in radish against bacterial leaf spot. The effect was due to the activity of specific biocontrol agents in the batches of composts that suppressed bacterial leaf spot. They identified *T. hamatum* (T382) as the most active inducer of (ISR) in radish. *T. harzianum* has been shown to induce systemic resistance to *Phytophthora capsici* in pepper seedlings raised from seeds treated with this biocontrol agent.

Some species of the genus *Trichoderma* have been used as biocontrol agents against various soilborne, foliar, and postharvest phytopathogenic fungal pathogens (Chet, 1990). Several attempts have been made to use *Trichoderma* spp. to control plant-parasitic nematodes. Windham *et al.* (1989) reported reduced egg production in the root-knot nematode *Meloidogyne arenaria* following soil treatments with *T. harzianum* (T-12) and *T. koningii* (T-8) preparations. Spiegel and Chet (1998) reported the reduction of *Meloidogyne javanica* infection with several isolates of *T. lignorum* and *T. harzianum*.

1.2.4 *Trichoderma* Mode of Action

Mechanisms by which *Trichoderma* spp. function are mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrient and inactivation of the pathogen's enzymes **(**Samules, 1996).

1.2.4.1 Mycoparasitism

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack

and subsequent penetration and killing of the host. *Trichoderma* spp. may exert direct biocontrol by parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of cell-wall-degrading enzymes (CWDEs), mostly chitinases, glucanases and proteases (Harman *et al*., 2004).

Mycoparasitism involves morphological changes, such as coiling and formation of appressorium- like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol (Howell, 2003). *Trichoderma* spp. attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* spp. is attached, it coils around the pathogen and forms the appresoria. The following step consists of the production of (CWDEs) and peptaibols (McIntyre *et al*., 2004), which facilitates both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. For mycoparasitism of Pythiaceous fungi, (β-1,4-glucanases) may also be important. To add even more complexity, peptaibol antibiotics are specifically produced by *T. harzianum* in the presence of fungal cell walls, and can probably be considered as part of the mycoparasitic complex (Schirmbock *et al.*, 1994).

1.2.4.2 Antibiosis

Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonistic microorganisms; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others were reported (Vey *et al.*, 2001).

The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Monte, 2001). Synergetic effects between an endochitinase from *T. harzianum* and gliotoxin, and between hydrolytic enzymes and peptaibols on conidial germination of *B. cinerea* is well known (Howell, 2003).

Antibiosis and hydrolytic enzymes during fungal interactions have been described by (Howell, 2003). When combinations of antibiotics and several kinds of hydrolytic enzymes were applied to propagules of *B. cinerea* and *Fusarium oxysporum*, synergism occurred, but it was lower when the enzymes were added after the antibiotics, indicating that cell-wall degradation was needed to establish the interaction (Howell, 2003).

1.2.4.3 Competition

Competition for space or nutrients has long been considered as one of the "classical" mechanisms of biocontrol by *Trichoderma* spp. (Elad *et al*., 1999). Competition for limiting nutrients results in biological control of fungal phytopathogens (Chet *et al*., 1979a). For instance, in most filamentous fungi, iron uptake is essential for viability.

Some *Trichoderma* spp. produces highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet and Inbar, 1994). For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* spp. according to iron availability. In addition, *T. harzianum* controls *F. oxysporum* by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Tjamos *et al.,* 1992).

Competition has proved to be particularly important for the biocontrol of phytopathogens such as *B. cinerea*, the main pathogenic agent during the pre- and post-harvest of crops in many countries (Latorre, 2001). The extraordinary genetic variability of this fungus makes it possible for new

strains to become resistant to essentially any novel chemical fungicide it is exposed to (Latorre, 2001). The advantage of using *Trichoderma* spp. to control *B. cinerea* is the coordination of several mechanisms at the same time, thus making it practically impossible for resistant strains to appear. The most important is nutrient competition, since *B. cinerea* is particularly sensitive to the lack of nutrients. *Trichoderma* spp.has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* spp. to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide-spread in fungal environments: cellulose, glucan and chitin among others, all of them rendering glucose (Chet *et al.*, 1997).

1.2.4.4 Enzymes

Lorito (1998) listed 10 separate chitinolytic enzymes produced by *T. harzianum* alone. Similar levels of diversity exist with β-1, 3-glucanases. In addition, β-1, 6-glucanases (Lora *et al.*, 1995) and proteases are likely involved. Enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of pathogen fungal cell walls, there by destroying cell wall integrity.

Woo *et al.* (1999) disrupted chitinase activity in *T. harzianum* (P1) and showed reduced biocontrol activity against *B. cinerea* on bean leaves. Lorito *et al*. (1998), who transferred the gene encoding endochitinase from *T. harzianum* (P1) into tobacco and potato, demonstrated a high level and broad spectrum resistance against a number of plant pathogens.

Kapat *et al*. (1998), suggested that biocontrol of *B. cinerea* by *T. harzianum* (T39), might be due, in part, to the actions of *T. harzianum* produced proteases that inactivated the hydrolytic enzymes produced by *B. cinerea* on bean leaves. The protease enzymes break down hydrolytic enzymes into peptide chains and/or their constituent amino acids and there by

destroy their capacity to act on plant cells. They further demonstrated that protease solutions produced by the biocontrol fungus on bean leaves partially deactivated hydrolytic enzymes and reduced disease severity by 56 to 100% when the solutions were used to treat leaves infected with the pathogen. They also showed that the addition of protease inhibitors to drops containing mixed conidia of the two fungi, applied to bean leaves, nullified the biocontrol effect of the *T. harzianum* conidia.

Migheli *et al.* (1998) showed that transformants of *T. longibrachiatum*, over-expressing a gene encoding 1, 4-endoglucanase, were slightly more effective in the biocontrol of *Pythium ultimum* on cucumber than the wild type. They concluded that a mixture of several enzymes might be necessary for efficient cell wall lysis.

The concept of enzyme biosynthesis as a mechanism of biocontrol has been expanded to include synergism between enzymes and antibiotics. Di Pietro *et al.* (1993) studied the synergistic effects of endochitinase and gliotoxin on conidial germination of *B. cinerea*. They found that the treatment of *B. cinerea* conidia with the combination was much more effective than treatment with the enzyme or the antibiotic alone. Schirmbock *et al.* (1994) noticed a similar effect on conidial germination and hyphal elongation of *B. cinerea* when the fungus was treated with combinations of hydrolytic enzymes and peptaibols produced by *T. harzianum*.

Lorito *et al.* (1996) further expanded this concept by combining a number of antifungal compounds with several kinds of hydrolytic enzymes and applying them to propagules of *B. cinerea* and *F. oxysporum*. Synergism occurred in all cases, but the level depended on the antifungal activity of the enzyme. Synergism was lower when the enzyme was added after the antifungal compound, indicating that cell wall degradation was needed in order to establish the interaction.

1.3 Study objectives

1) Recover new Palestinian native isolates of *Trichoderma* from agricultural areas in the West Bank.
2) Assess the potential pathogenicity of *Trichoderma* native isolates against early blight of tomato caused by *A.solani*.
3) Test the most promising isolates in different concentrations, formulations, and under various temperatures.
4) Study the mode of action of these local isolates and the way they function against the pathogen.
5) Compare biological control with the most commonly used fungicides against early blight in an integrated approach.
6) Field evaluation of the selected native strains of the biocontrol agent.

Chapter Two

2. Materials and Methods

2.1 Collection of soil samples

One hundred and twenty soil samples were collected from various agricultural fields from four districts in the West Bank (Hebron, Jenin, Qalqelia and Tulkarim) during the summer of 2004. Soil samples were collected at the depth of 5-10 cm from soil surface. The samples were collected from open fields of irrigated vegetables. Each soil sample was placed in a plastic bag, and then mixed thoroughly and manually by external manipulation.

2.2 Isolation of *Trichoderma* species from soil

Trichoderma species were isolated from soil using dilution plate technique. Twenty five grams of each sample were suspended in 250 ml of 0.1% water agar. Samples were shaked for 30 minutes on a rotary shaker at 200 rpm. Dilutions of 10^{-1}, 10^{-2} and 10^{-3} were made for each soil sample and an aliquot of 0.1 ml of 10^{-3} soil suspension was pipetted into 90 mm diameter Petri dishes and spread on *Trichoderma* –selective media (TSM) surface with a glass rod (Elad *et al*., 1981). Five Petri dishes (replicates) were used for each soil sample and suspension concentration. The plates were incubated for 7days at 25ºC. After incubation, *Trichoderma* spp. colonies on the surface of (TSM) were transferred to potato dextrose agar medium (PDA) for purification and further identification.

Worth mentioning that in addition to the *Trichoderma* spp. isolates managed to be isolated in this study, 47 isolates of *Trichoderma* species were used from the already existing collection of Prof. Barakat of Hebron University.

2.3 Growth and reproduction studies

2.3.1 Mycelia growth rate (MGR)

The method was based on that of Elad *et al* (1981). Mycelium growth rate of *Trichoderma* isolates was assessed on plates containing potato dextrose agar (PDA) according to the following procedure: four Petri dishes (90mm diameter) containing PDA were centrally inoculated with mycelial disk (5mm) of agar plugs from 7-day-old PDA cultures of each *Trichoderma* isolate to determine the average mycelial growth rate (MGR) for each isolate. Plates were incubated at 25°C under continuous light and inspected daily for three consecutive days. Mycelial growth rate was recorded every 24 hours during this period. The colony diameter was measured as the mean of two perpendicular diameters measured at the third day minus the diameter at first day. Mycelium growth rate (MGR) was calculated by using the formula (Elad *et al.*, 1981):

MGR cm^2/day = (((D2-D1) ^{2} /400)*3.14)) (T2-T1) (day)

Where **D2**: Diameter in second evaluation (mm), **D1:** Diameter in first evaluation (mm), **T2:** Time of the second evaluation and **T1**: Time of the first evaluation (day). The experimental design used was completely randomized with four replicates (plates) used for each treatment.

2.3.2 Sporulation

Seventy three native isolates of *Trichoderma* spp. were grown on potato dextrose agar at 25°C under continuous light. Mycelial disk (5mm) was taken from each plate (replicate) of 7day - old cultures and homogenized in 10 ml of alcohol**.** The resulting conidial concentration in the suspension was determined by haemacytometer under microscopic power of 40X. Spores productions perunit area of the plate was calculated by using the formula:

Sporulation = (Mean*6.25*10^6*10ml) *1/area of the disk.

The experimental design used was completely randomized with four replicates (plates) for each treatment**.**

2.3.3 Effect of temperature on growth parameters (MGR and spore's production)

The effect of temperatures on mycelial growth of the *Trichoderma* isolates (Jn14, Jn17, T34, and Jn58) on PDA under continuous light was studied. Ninety mm diameter Petri dishes were inoculated with (5mm) agar plugs from 7-day-old PDA cultures of the isolates. Plates were then incubated at different temperatures including 15, 20, 25, 30, and 35°C respectively, with five replicates for each treatment under light and dark conditions. Mycelium growth was measured as the mean of two perpendicular diameters after 24 hours and 48 hours of incubation. Mycelium growth rate was calculated in (cm^2 /day) as mentioned earlier.

At the end of mycelia growth study, the plates of all *Trichoderma* isolates were incubated for 3 more weeks and used for the assessment of spore production. Mycelial disks (5mm) were taken from each plate (replicate) after 3, 5, 7, 8, 10, 12 and 15 days from inoculation under light and dark condition and homogenized in 10 ml of alcohol. The resulting spore concentrations were counted using a hemocytometer under microscopic magnification power of 40X. The experimental design was completely randomized with five replicates (plates).

2.4 *Alternaria solani* isolate

A. solani isolate used in the experiments was isolated from diseased tomato plant collected from a field in the Hebron area. Diseased leaves stem and fruit were cut into 4-5 mm pieces, surface–sterilized by immersion in 1% sodium hypochlorite solution for 5-7 min, and rinsed three times with sterile distilled water. Three pieces were placed in each 90 mm Petri dish containing potato dextrose agar (PDA) medium supplemented with 250 mg/l

chloramphenicol (Difco). The plates were incubated for 7 days at 25ºC. Fungal hyphae of *A. solani* were sub-cultured on potato dextrose agar medium as single spore cultures. Plates were then incubated for 12-15 days at 25ºC under continuous light.

2.5 Biological control studies

2.5.1 Preliminary evaluation of antagonistic potential (Bioassays)

The antagonistic potential of *Trichoderma* spp. isolates was studied using the method of (Mihuta and Rowe, 1986). Seventy- three native isolates of *Trichoderma* spp. were grown on potato dextrose agar (PDA) at 25°C under continuous light. Conidia were harvested from cultures after 7 days by flooding the plates with 10 ml of sterile distilled water (SDW) to get final concentration of *Trichoderma* spores of $4*10^6$ /ml (CFU). Eight- week-tomato plants (*Lycopersicon esculentum*, L., cv. Facultia 16/84) were sprayed (40 ml/plant) with *Trichoderma* - spore suspension 24 hours before inoculation with *A. solani* suspension. *A. solani* used in the inoculation was previously isolated from naturally infected tomato leaves maintained and grown on potato dextrose agar.

Conidial suspension of *A. solani* were prepared from 15-days old cultures by harvesting conidia from sporulating cultures in SDW and filtered through muslin. Spore concentration was determined with a hemocytometer and adjusted as ($4x10^5$spore/ml). Additives of 1% glucose and 1% KH_2PO_4 were added to spore suspension (O'Neill, *et al*., 1996). *Trichoderma* treated plants were then inoculated by micro sprayer with conidial suspension of *A. solani* (30ml/ plant). Plants were covered with transparent polyethylene bags and incubated in a growth room at 25ºC and a 12h photoperiod. Disease severity was evaluated 15 days after incubation as disease severity (%) according to a disease severity scale designed for this purpose Table (2.1).

Table (2.1): Disease severity scale

Disease (%)	Scale
0-20%	1-10 spots on leaves
20-40%	10-20 spots on leaves and spots starts to appear on stems
40-60%	20-30 spots on leaves and 10 spots on stems
60-80%	30-40 spots on leaves and 20 spots on stems
80-100%	Completely blighted plant

2.5.2 *Trichoderma* isolates Identification

The most promising *Trichoderma* isolates (Jn14, Jn17, Jn58 and T34) were taxonomically identified. *Trichoderma* isolates were grown on PDA in 90-mm Petri dishes, and plates incubated at 25°C for 7 days. Isolates were placed into groups according to colonial morphology, conidiation color and color of reverse colony (Bissett, 1991 a, b, c). Sterile glass cover slips, 50 x 25 mm, were held with forceps and immersed in autoclaved melted water agar (20g Difco agar / 1L distilled water) at about 45°C for 1-2 second, and allowed to drain. The cover slip was then laid singly on the surface of 2% solidified water agar in center of 90-mm diameter Petri dish, so that a thin film of agar set on the upper surface (Laing and Deacon, 1991). Each plate was inoculated with a 5 mm plug cut from the edge of 7 days old growing colonies of each *Trichoderma* isolate on potato dextrose agar (PDA). The plug was then placed 2cm apart of the cover slip placed on the agar surface,

so that *Trichoderma* spp. colony would grow across the coated cover slips. Plates containing coated cover slips were incubated at 25°C and inspected daily for four days for *a* mycelium growth. Each cover slip was removed carefully without damaging the fungus mycelium, and was then inverted on sterile microscopic slide (24.4 x 76.2mm) and sealed by nail varnish to prevent drying. The growing isolates were studied using fresh direct mounts in Lactephenol cotton blue under medium and high magnifications, x20, and x40, respectively. The top of the cover slip was cleaned, and microscopic observations were made throughout the coated cover slip and thin film of agar. Three replicates were used for each *Trichoderma* isolate.

2.5.3 Formulation studies

2.5.3.1 Spore suspension

Two isolates of *Trichoderma harzianum* (Jn14 and Jn58) were grown on (PDA) at 25°C under continuous light. Conidia were harvested from cultures after 7 days by flooding the plates with 10 ml of sterile distilled water to a get final concentration of *Trichoderma* spores $4*10^6$ /ml (CFU). Tomato plants were sprayed (40 ml/plant) with spore suspension 24 hours before inoculation with *A. solani* suspension. Conidial suspension of *A. solani* were prepared from 15-days old cultures by harvesting conidia from sporulating cultures in SDW and filtered through muslin. Spore concentration was determined with a hemocytometer and adjusted to ($4x10^5$spore/ml). Additives of 1% glucose and 1% KH_2PO_4 was added to the spore suspension. *Trichoderma* treated plants were inoculated by using micro sprayer containing conidial suspension of *A. solani* (30 ml/ plant). Plants were then covered with transparent polyethylene sacks and incubated in growth room at 25°C and a 12h photoperiod. Disease severity was evaluated 15 days after incubation as disease (%) according to the scale in (Table 2.1).

2.5.3.2 Talc formulation

The *T. harzianum* isolates (Jn14 and Jn58) was grown on 90 mm PDA medium plates for 7 days. Autoclaved and sealed Erlenmeyer flasks (250ml) containing 100 ml potato dextrose broth (PDB, Difco) were inoculated with 5mm mycelium plug of the isolates (Jn14 and Jn58). The flasks were fitted on a shaker at 200 rpm and incubated at 25ºC in a growth room for 15 days. The suspension was filtered through sterile glass funnel (max. pore size: 40-100 μm), and the mycelia mat discarded. The filtrate suspension containing spores was centrifuged at 5000 rpm for 15 mins and the supernatant was discarded. The spore pellets were washed with adding sterile distilled water, vortexes to ensure homogeneous suspension and centrifuged as discussed earlier (Repeated 2 times). After washing, the pellets were recovered in sterile water and the final volume was brought up to 100 ml. The 100 ml spore suspension was added to 200 ml of talc powder (Sigma, T-2015) and stirred with a sterile spatula. The mixture was placed on aluminum foil in a sterile tray (in the form of small heaps) in a disinfected aerated oven at 20ºC for 48-72 hours. Once dried, the heaps looked like "cakes" were crushed and sieved (200 μm) under sterile conditions to avoid contamination. The talc-formula can be stored dried in tightly closed flasks either at 4ºC or at room temperature. The density was evaluated by counting the number of (CFU/g) of talc using dilution plate technique; the concentration of conidia in talc was $5*10^8$ CFU/g for the isolate (Jn14) and $4*10^8$ CFU/g for the isolate (Jn58).

2.5.3.3 Formulation and disease severity

Eight week-old tomato plants in the flowering stage were sprayed with *T. harzianum* (Jn14 and Jn58) as spore suspension and talc formulation. The conidia concentration in talc was calibrated by using dilution plate technique and in spore suspension by using the haematocytometer. The plants were treated by talc or spore suspension at concentration of 10^7 and 10^8 CFU/ml.

For each concentration, 5 plants (replicates) were used in a completely randomized design. *Trichoderma* treated plants were inoculated with conidial suspension of *A. solani* after 24 hour by a micro sprayer (30ml/plant) at the concentration of $4*10^5$ CFU/ml. The control treated plants (without pathogen) were sprayed with sterile distilled water. Plants were covered with transparent polyethylene bags and incubated in growth room at 25ºC and 12h photoperiod. Disease was evaluated after 15 days of incubation and expressed as disease % (Table 2.1).

2.5.4 Bioagent's time of application

The most effective isolate of *T. harzianum* (Jn14) was grown on potato dextrose agar (PDA) at 25°C under continuous light. Conidia were harvested from cultures after 7 days by flooding the plates with 10 ml of sterile distilled water and removed with sterile bent glass rod .The resulting conidial concentration in the suspension was determined by the haemacytometer under microscopic magnification power of 40X . Sterile distilled water was added to bring the concentration to $4*10^6$ conidia / ml (CFU).

Eight weeks-old tomato plant were sprayed (40ml/plant) with spore suspension of *Trichoderma*. Conidial suspension of *A. solani* was then prepared from 15 days old cultures by harvesting conidia from sporulating cultures in SDW and filtered through muslin. Spore concentration was determined and adjusted to ($4x10^5$spore/ml). Additives of 1% glucose and 1% KH_2PO_4 were added to the spore suspension. *Trichoderma* treated plants were then inoculated with the conidial suspension of *A. solani* (30 ml/ plant).

The experiment involved the following treatments:

(1) Plants treated with *Trichoderma* 24 hours before inoculation with *A. solani* (B 24).

(2) Plants treated with *Trichoderma* and *A. solani* at the same time (0).

(**3)** Plants treated with *Trichoderma* 24 hours after inoculation with *A. solani* (A 24).

(**4)** Plants treated with *Trichoderma* 48 hours after inoculation with *A. solani* (A48).

Treated plants were then covered with transparent polyethylene sacks and incubated in growth room at 25°C. Disease severity was evaluated 2 weeks after incubations as disease % (Table 2.1).The experimental design was completely randomized with five replicates.

2.5.5 Bioagent's inoculum concentration

The effect of different *T. harzianum* (Jn14 and Jn58) conidial concentrations on disease severity was evaluated. Eight weeks older, tomato plant were sprayed (40 ml/plant) with *Trichoderma* spore suspensions (10^5, 10^6, 10^7 and 10^8 CFU/ml) using a microsprayer. (24) hours later, treated plants were sprayed with *A. solani* conidial suspension (30 ml/ plant) spore concentration was determined and adjusted to ($4x10^5$spore/ml). Additives of 1% glucose and 1% KH_2PO_4 were added to conidial suspension using a similar microsprayer. Plants were then covered with transparent polyethylene sacks and incubated in a growth room at 25°C and 12h photoperiod. Disease severity was evaluated 15 days after incubation (Table 2.1)

2.5.6 Bioagent's mode of action

2.5.6.1 Effect of *Trichoderma harzianum* metabolites on mycelial growth of *A. solani*

The ability of *T. harzianum* isolates, (Jn14, T34, Jn17 and Jn58) to inhibit the mycelium growth of *A. solani* through the production of fungitoxic metabolites at 25ºC was tested according to the method of (Dennis and Webster, 1971c). Fifty ml of potato dextrose broth (PH=6) in 250 ml Erlenmeyer flasks, was inoculated with 5 mm- mycelial disks from 7 days old

cultures of the four *Trichoderma* isolates (Jn14, T34, Jn17 and Jn58) and incubated at 25ºC. After 15 days of incubation, the cultures were filtrated through Millipore membrane filter (0.45µm) and were autoclaved at 121°C for 30 minutes. The culture filtrates were then added to PDA at 10% and 25% (v/v). The filtrate amended PDA plates were then centrally inoculated with 5 mm mycelial discs of *A. solani*. Plates were incubated at 25ºC with three replicates; unamended PDA served as control. The mycelium growth rate of *A. solani* was measured after 3-7 days as cm^2 /day and percent inhibition was calculated. The experimental design was completely randomized with three replicates (plates) for each treatment.

2.7 Chemical control studies

Fungicides

Five fungicides, Antracol® 70% W.P (Propineb), Daconile® 75% W.P (Chlorothalonil), Manzidan® 80% W.P (Mancozeb), Rovral® 50% W.P (Iprodione), and Manbegan® 80% W.P (Maneb), were used for evaluating their efficiency against *A. solani in vitro* and *in vivo* Table (2.2).

Table (2.2): Fungicides

Fungicides	Trade name	Producer	Composition
Propineb	Antracol® 70% W.P	Bayer	Zinc- propylenebisdithio carbamate
Chlorothalonil	Daconile® 75% W.P	Bayer	Tetrachloro isophthalonitrils
Mancozeb	Manzidan® 80% W.P	Macktashem	Manganese ethylenebis (dithiocarbamate) (polymeric) complex with zinc salt.
Iprodione	Rovral® 50% W.P	Bayer	3(3,5-Dichlorophenyl)-N-isopropyl-2,4-dioxoimidazoldine-l-carboxamide.-
Maneb	Manbegan® 80% W.P	Agan	Manganese ethylenebis(dithiocarbamate)(polymeric)

2.7.1 Effect of fungicides on *Alternaria solani* mycelial growth

This experiment was conducted to evaluate the effect of different concentrations of the fungicides mentioned above on mycelium growth rate of *A. solani*. Five different concentrations of fungicides (0.5, 1, 3, 5 and 7 µg ml^{-1}) were studied. Each concentration was added to 100 ml PDA medium in an Erlenmeyer flask; the mixture was then poured into 90 mm Petri dishes. All Petri dishes were inoculated with 5mm- mycelial disks of 12-day old cultures of the fungus *A. solani*. Following inoculation mycelial growth rate of *A. solani* was measured after 3-5 days as cm^2 /day. Plates were incubated at 25°C under continuous light. The experimental design was completely randomized with five replicates per each concentration for each fungicide. Mycelium growth rate were calculated by using the formula:

MGR cm^2/day = (((D2-D1) ^{2} /400)*3.14)) (T2-T1) (day).

Where **D2**: Diameter in the second evaluation (mm), **D1:** Diameter in the first evaluation (mm). **T2:** Time of the second evaluation and **T1:** Time of the first evaluation (day).

2.7. 2 Effect of fungicides on *Alternaria solani* spore's germination

This experiment was conducted to evaluate the effect of the fungicides, Propineb and Iprodione on spore's germination of *A. solani* at the concentrations of (0.5, 1 and 3µg ml^{-1}). Each concentration was added with 100 ml PDA medium in an Erlenmeyer flask; the mixture was then poured in Petri dishes, All Petri dishes were inoculated with conidial suspension of *A. solani* (200µ/plate) with spore concentration of (10^3spore/ml). Plates were incubated at 25°C under continuous light for 3 days before measuring spore's germination. The experimental design was completely randomized with five replicates.

2.7.3 Effect of fungicides on *Alternaria solani* disease severity

Eight weeks - tomato plants were sprayed (30 ml/plant) with *A. solani* spore suspension ($4x10^5$ spore/ ml). After 8 days, inoculated plants were sprayed with the following fungicides: Propineb, Chlorothalonil, Mancozeb, Iprodione and Maneb at the concentrations of (1 and 3 µg ml^{-1}). Each plant was sprayed with 40 ml of the fungicide suspension. The control treatment plants were sprayed with *A. solani* alone. Each treatment was replicated five times and treated plants were incubated in a growth chamber at 25°C. Disease severity was evaluated after 15 days of incubation as disease (%) according to the scale developed earlier (Table 2.1).

2.7.4 Effects of fungicides on *Trichoderma harzianum* spore's germination

This experiment was conducted to evaluate the effect of the fungicides (Propineb and Iprodione) on spore's germination of *Trichoderma harzianum* isolates (Jn14 and Jn58) at the concentrations of (0.5, 1 and 3 µg ml^{-1}). Each concentration of the above fungicides was added to 100 ml PDA medium in an Erlenmeyer flask; the mixture was then poured in Petri dishes. All Petri dishes were inoculated with conidial suspension of *T. harzianum* Jn14 and Jn58 (200µ/plate).

Conidia were harvested from 7-day old cultures by flooding the plates with 10 ml of SDW to get a final concentration of $4*10^6$/ml (CFU). Plates were incubated at 25°C under continuous light for 2 days before measuring germination. The experimental design was completely randomized with five replicates.

2.8 Integrated control studies

2.8.1 *In vivo* studies

The effect of combination of *T. harzianum* (Jn14) and the fungicides Propineb and Iprodione (3µg ml^{-1}) on disease severity was studied. Eight weeks - tomato plants were sprayed (30 ml/plant) with *A. solani* suspension at the concentration of ($4x10^5$spore/ml). After 7 days, inoculated plants were sprayed with the fungicides Propineb and Iprodione (40ml/plant); the control treated plants were sprayed with *A. solani* alone. After 10 days, tomato plants were sprayed (40 ml/plant) with spore suspension of *T. harzianum* at the concentration of 10^8 conidia/ ml.

Accordingly the experiment involved the following treatments:

(1) *T. harzianum* alone **(2)** Combination of *T. harzianum* and fungicides **(3)** Fungicides alone **(4)** Control (*A.solani* alone). Treated plants were then covered with transparent polyethylene bags and incubated in growth chamber

at 25°C. Disease severity was evaluated 2 weeks after incubation as disease (%). The experimental design was randomized complete block design with five replicates (plants) for each treatment.

2.7.2 Field application

The experiment was conducted at Al – Aroub agricultural station (10Km North of Hebron). An open field was transplanted with tomato plants, grown in rows and drip irrigated (120X50 cm). After 10 weeks from transplanting, tomato plants were sprayed with (100 ml/plant) of *A. solani* suspension at the concentration of ($4x10^5$spore/ml). After 15 days, the plants were sprayed with the fungicides Propineb and Iprodione (150 ml/plant) at the concentration of 3µg ml^{-1} ; the control treated plants were sprayed with *A. solani* only. After 5 days, tomato plants were sprayed (200 ml/plant) with spore suspension of *T. harzianum* (Jn14) at the concentration of 10^8 conidia/ ml.

The experiment involved the following treatments:

(1) *T. harzianum* alone **(2)** Combination of *T. harzianum* and fungicides **(3)** Fungicides alone **(4)** Control (*A. solani* alone). Disease severity (%) was evaluated after 15 days. The experiment design was randomized complete block with eight replicates (plants) for each treatment.

Chapter Three

3. Results

3.1 Isolation of *Trichoderma* species from soil

Twenty six isolates of *Trichoderma* spp. were recovered from 120 soil samples collected from different locations in the West Bank during the summer of 2004. The highest number of isolates (15) was recovered from Jenin followed by six isolates recovered from Tulkarem. Three isolates were recovered from Hebron and two from Qalqelia. The *Trichoderma* species isolates were recovered from soils planted with various vegetables and fruit trees (Table 3.1).

Table (3.1): *Trichoderma* spp. isolates recovered from soil samples collected from different locations in the West Bank (2004).

Area	Number of isolates recovered	Native *Trichoderma* isolates recovered	Site	Cultivation (irrigated vegetable)
Jenin	4	Jn48, Jn49, Jn50, Jn51	Bier Albasha	Tomato, pepper, cucumber
	3	Jn52, Jn53, Jn54	Kufrazan	Tomato, eggplant
	3	Jn55 , Jn56, Jn57	Burken	Tomato, cucumber, Squash
	2	Jn58, Jn59	Qabatya	Tomato, cucumber
	2	Jn60, Jn61	Sanor	Tomato
	1	Jn62	Bet kad	Pepper
Tulkarem	3	T63, T64, T65	Romana	Tomato, Bean
	2	T66,T 67	Al-Jarba	Pepper, Tomato
	1	T68	Attel	Pepper
Hebron	3	H69, H70, H71	Albaka	Tomato, Grape
Qalqilya	2	Q72, Q73	Qalqilya	Pepper, Tomato

3.2 Growth and reproduction studies

3.2.1 Mycelia growth rate (MGR)

The mean of mycelia growth rate for the isolates ranged from 6.0 cm^2/day for Jn19 and 24.1 cm^2 /day for Jn14. Results of mycelial growth rate of the *Trichoderma* isolates grown on PDA are presented in (Figure 3.1). The mycelia growth rate in (cm^2 /day) for *Trichoderma* isolates varied according to the isolates tested. The highest mycelium growth rate was 24.1 cm^2 /day recorded by *T. harzianum*, isolate Jn14.

Figure **(**3.2**)** shows the mean of mycelia growth rate for the rest of isolates tested. In this batch, *T. harzianum*, isolate Jn58 recorded the highest growth rate (22 cm^2 /day); the lowest was recorded by the isolate H69 (5 cm^2 /day).

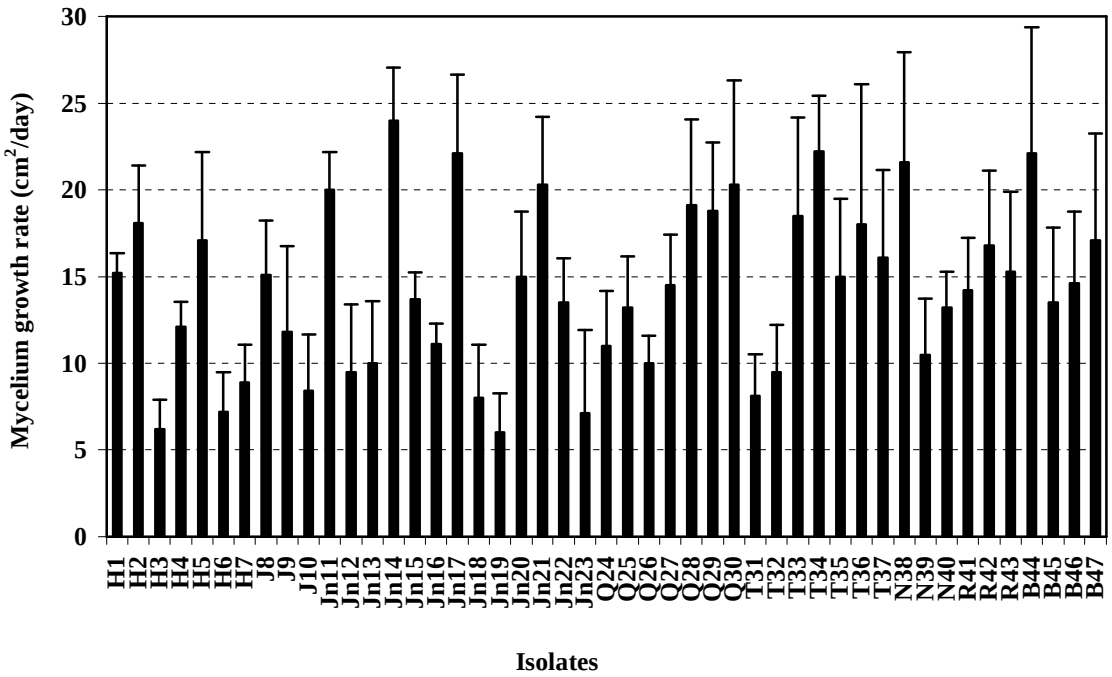


Figure (3.1): Mycelia growth rate of *Trichoderma* isolates growing on PDA medium incubated under light conditions at 25 °C.

(LSD =5.6, P ≤ 0.05)

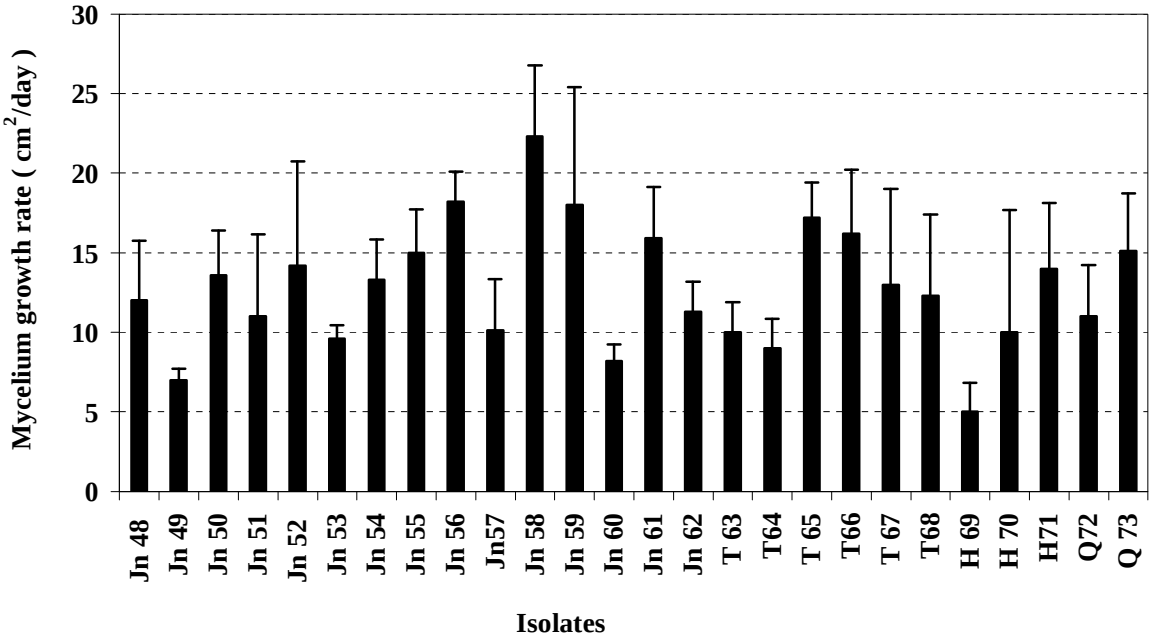


Figure (3.2): Mycelia growth rate of *Trichoderma* isolates growing on PDA medium incubated under light conditions at 25 °C.

(LSD = 6, P ≤ 0.05).

3.2.2 Sporulation

Sporulation of the isolates ranged from $3x10^8$ spores /cm^2 for the isolate (H3) and $10x10^8$spore /cm^2 for isolate (Jn17). Results of the sporulation of *Trichoderma* spp. isolates grown on PDA medium and incubated under light condition at 25°C are presented in (Figure 3.3). The highest sporulation was $10x10^8$ spore /cm^2 recorded by the isolate (Jn17).

Figure (3.4) shows the mean rates of sporulation for the rest of isolates tested. In this batch, *T. harzianum*, isolate Jn58 recorded the highest sporulation ($9.6 x10^8$ spore /cm^2); the lowest was recorded by the isolate H69 ($1.4x10^8$ spore /cm^2).

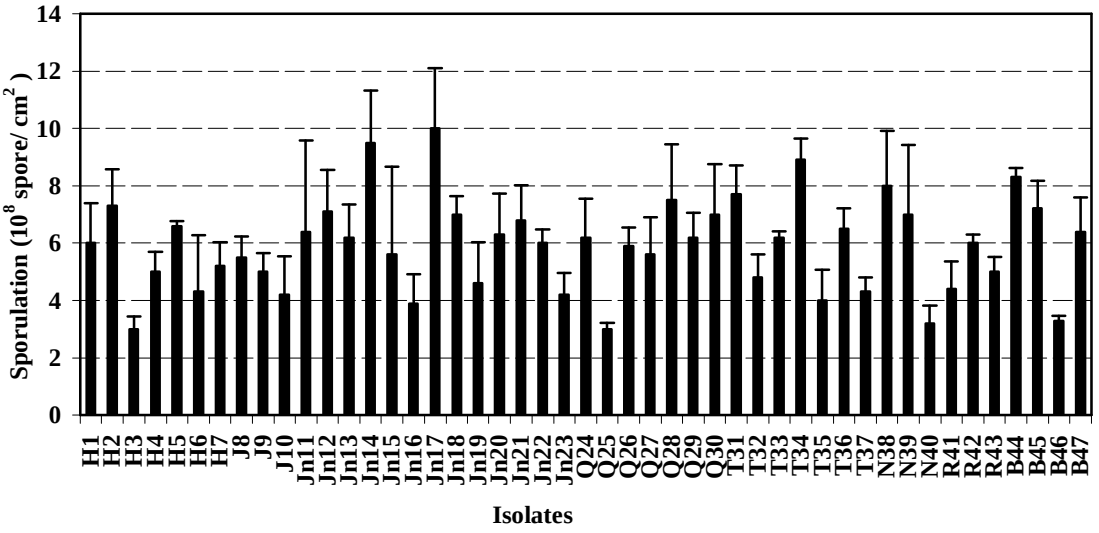


Figure (3.3): Sporulation of *Trichoderma* spp. isolates (10^8spore/cm^2) after 7 days of growing on PDA medium and incubated under light conditions at 25 °C. (LSD = 2, P ≤ 0.05)

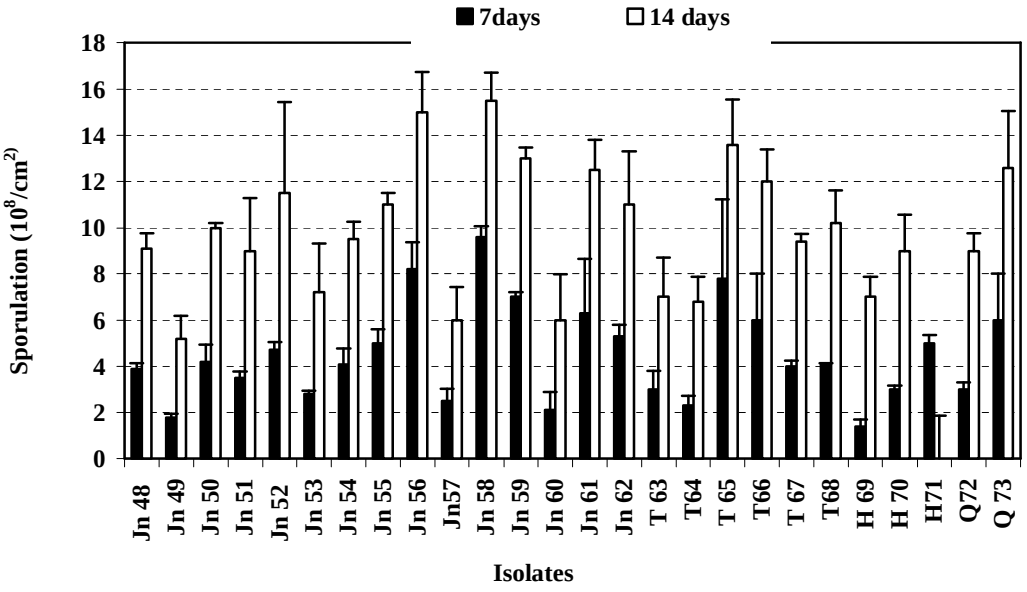


Figure (3.4): Sporulation of *Trichoderma* spp. isolates (10^8 spore/cm^2) after 7 and 14 days of growing on PDA medium and incubated under light conditions at 25 °C. (LSD = 2, P ≤ 0.05)

3.2.3 The effect of temperature on growth parameters (MGR and spore's production)

Mycelium growth rate and spore's production of *T. harzianum* isolates (Jn14, Jn58 and T34) and *T. atroviride* (Jn17) were measured under five temperatures (15, 20, 25, 30 and 35°C) with 5 replicates on PDA under light and dark conditions.

The results (Figure 3.5) showed that the four *T. harzianum* isolates and *T. atroviride* reached a peak in mycelial growth rate at 25°C and was completely inhibited at 35°C. The variability between the isolates was clearly observed at various temperatures especially when light was a factor. The isolate (Jn14) recorded a growth of (42 cm^2 /day) at 25°C under dark and was clearly the highest while the lowest growth (0.34 cm^2 /day) was recorded by the isolate (T34) at 35°C under light. It's worth mentioning as well that MGR was higher under dark than under light condition for all isolates and under all temperature schemes with some variation in statistical significance.

The results of sporulation rates (Figure 3.6) showed that the four *Trichoderma harzianum* isolates and *Trichoderma atroviride* sporulation of the isolate (Jn58) reached a peak at 25°C and was almost completely inhibited at 35°C. The isolate (Jn58) recorded a sporulation of ($13*10^7$ spore /ml) at 25°C under light and was clearly the highest while the lowest sporulation ($1*10^7$ spore /ml) was recorded by the isolate (T34) at 35°C under dark. It's worth mentioning as well that spore's production was higher under light than under dark condition for all isolates and under all temperatures schemes with some variation in statistical significance.

Studying the rate of sporulation of the isolates Jn14 and Jn58 with time (Figure 3.7) showed that the rate of sporulation for both isolates was higher under light. Both isolates sporulation increased with time at the optimum

temperature, reaching a peak of ($12*10^7$ spore /ml) and ($14*10^7$ spore /ml), respectively after 15 days.

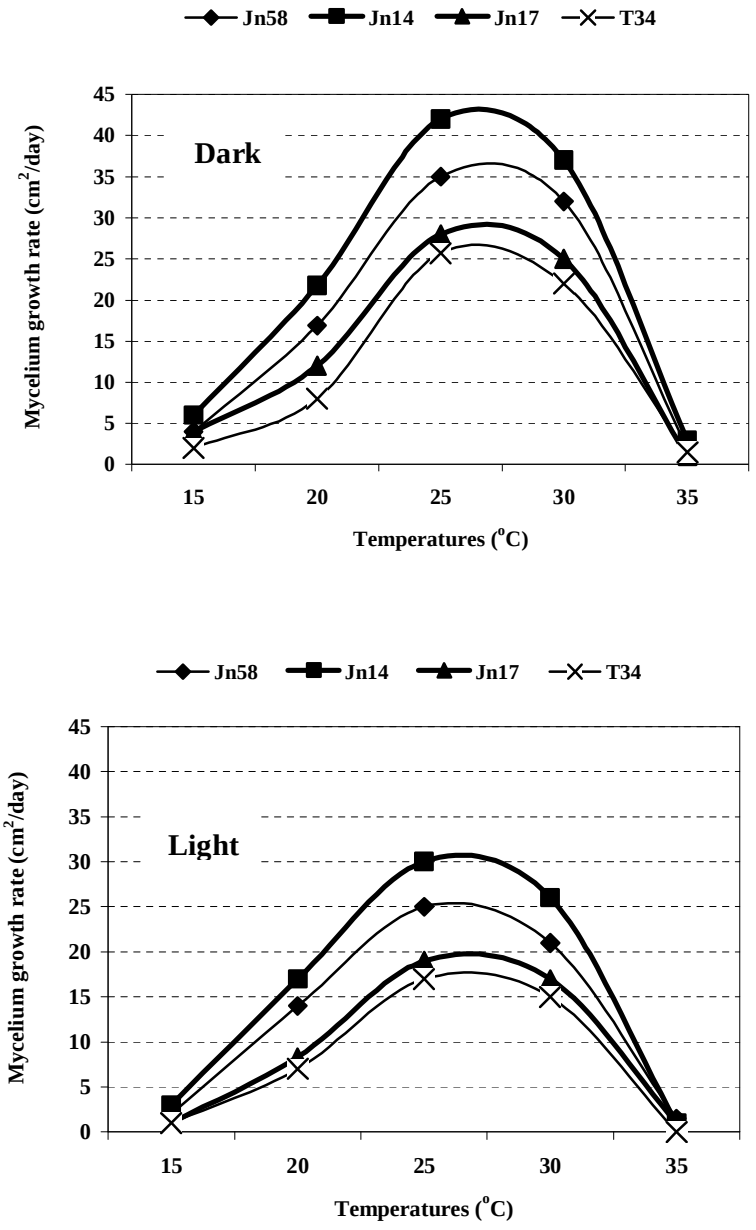


Figure (3.5): Mycelia growth rate (cm^2/day) of most promising native *T. harzianum* (Jn14, T34 and Jn58) and *T. atroviride* (Jn17) growing on PDA medium and incubated at different temperatures 15, 20, 25, 30 and 35°C in (Light and Dark) after 3 days. (LSD =11, $P \leq 0.05$).

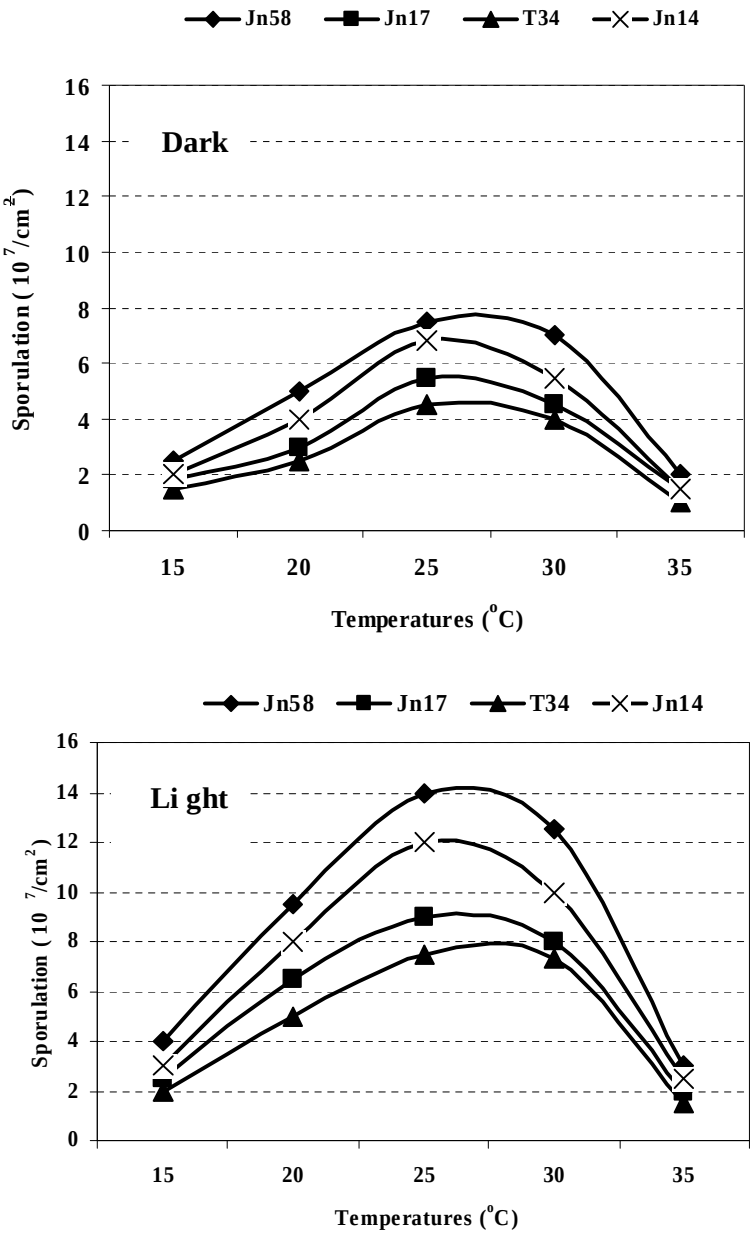
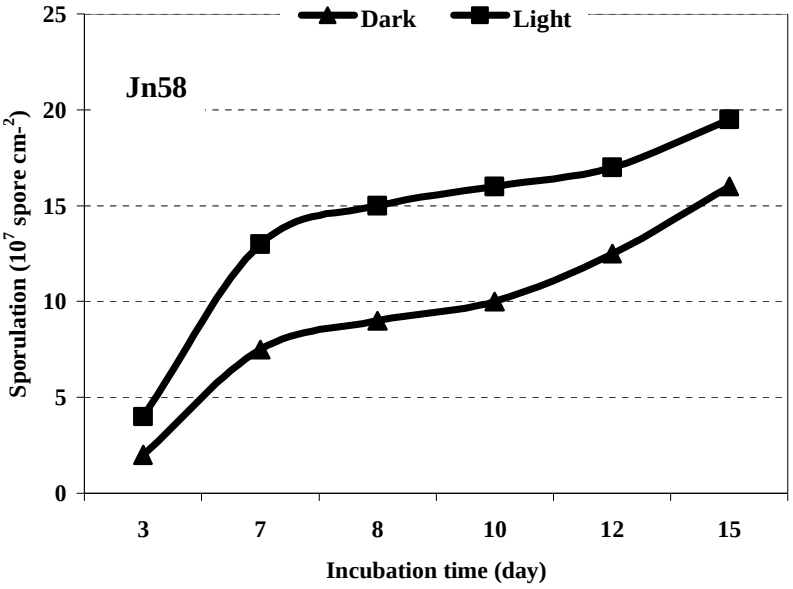


Figure (3.6): Sporulation of most promising native *T. harzianum* (Jn14, T34 and Jn58) and *T. atroviride* (Jn17) growing on PDA medium at different temperatures 15, 20, 25, 30 and 35°C under (Light and Dark) after 7 day. (LSD =2, P ≤ 0.05).



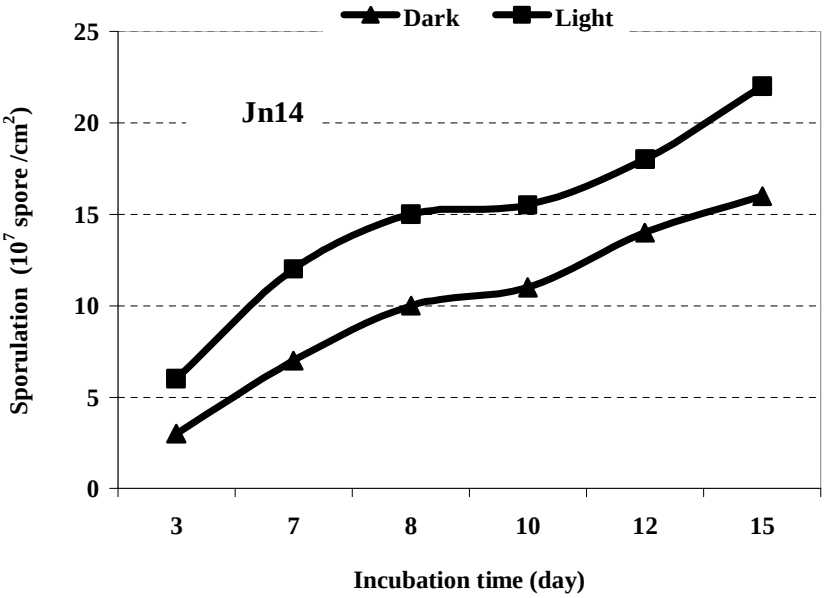


Figure (3.7): Sporulation of *T. harzianum* isolates (Jn58 and Jn14) growing on PDA medium at 25°C under light and dark with time (days). Jn58 (LSD = 3, P ≤ 0.05) and Jn14 (LSD = 2, P ≤ 0.05)

3.3 Biological control studies

3.3.1 Evaluation of antagonistic potential (Bioassays)

Seventy three isolates of *Trichoderma* were tested as antagonists of *A. solani* (early blight) on tomato plants. The tested *Trichoderma* isolates reduced early blight of tomato plants in different rates. The preliminary bioassays done on disease reduction by isolates (Figure 3.8 and 3.9), revealed a range of disease reduction from 79% recorded by the isolate (Jn14) and 76% recorded by the isolate (Jn58). In this batch of antagonistic potential bioassays, the isolates (Jn14, Jn17, T34, Q28, B44, Jn58, Jn56, T65, Jn59, Jn21, Jn11, N38, and T31) (Figure 3.8 and 3.9) recorded the highest values of disease reduction compared to the control.

The preliminary results obtained from the first batch of antagonistic potential bioassays were the base for a second batch (II) testing (Figure 3.10). Results obtained reconfirmed the antagonistic potential of (13) *Trichoderma* spp. isolates. Disease reduction in this batch ranged from 26% and 83% compared to the control.

The *Trichoderma* spp. isolates that recorded the highest disease reduction rates (Jn14, Jn58, Jn17 and T34) in batch 2 was further tested in a third batch (III) of bioassays (Figure 3.11). Results showed that Jn14 recorded again the highest disease reduction rate (72 %) followed by Jn58 (60%), Jn17 (54 %) and finally T34 (42 %).

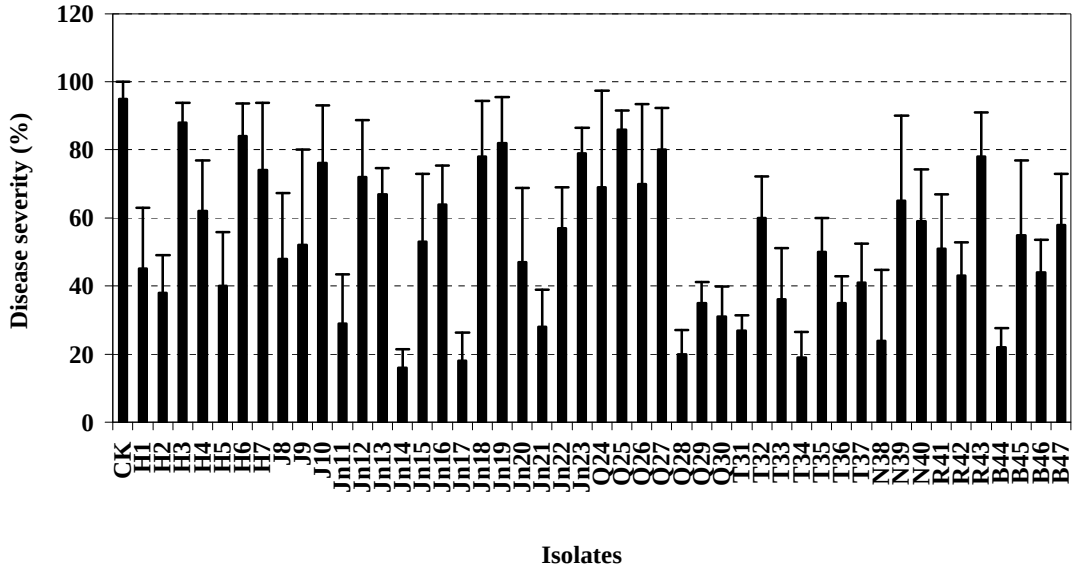


Figure (3.8) Batch I: Effect of *Trichoderma* spp. isolates on early blight disease severity (%) after 15 days incubated at 25 °C.

(LSD= 18.4, P ≤ 0.05)

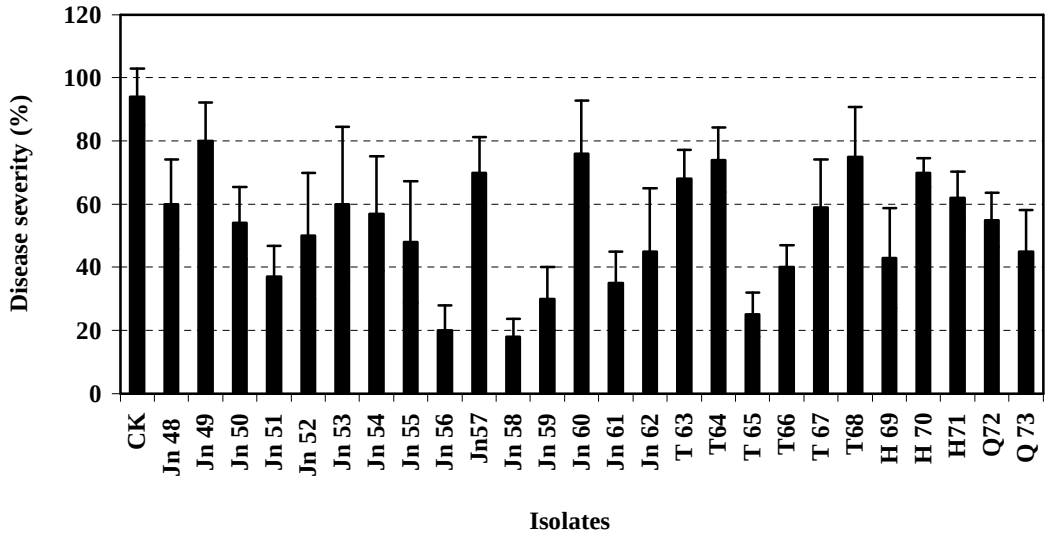


Figure (3.9) Batch I: Effect of *Trichoderma* spp. isolates on early blight (*A. solani*) disease severity (%) after 15 days incubated at 25°C.

(LSD =16.7, P ≤ 0.05).

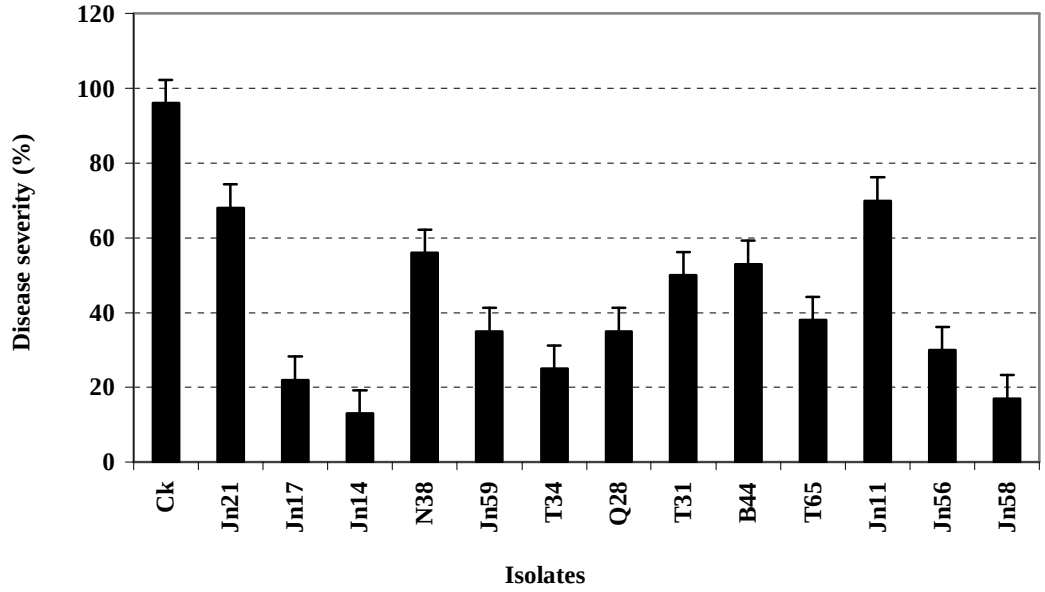


Figure (3.10) Batch II: Effect of *Trichoderma* spp. isolates on early blight disease severity (%) after 15 days incubated at 25 ºC.

(LSD =14, P ≤ 0.05)

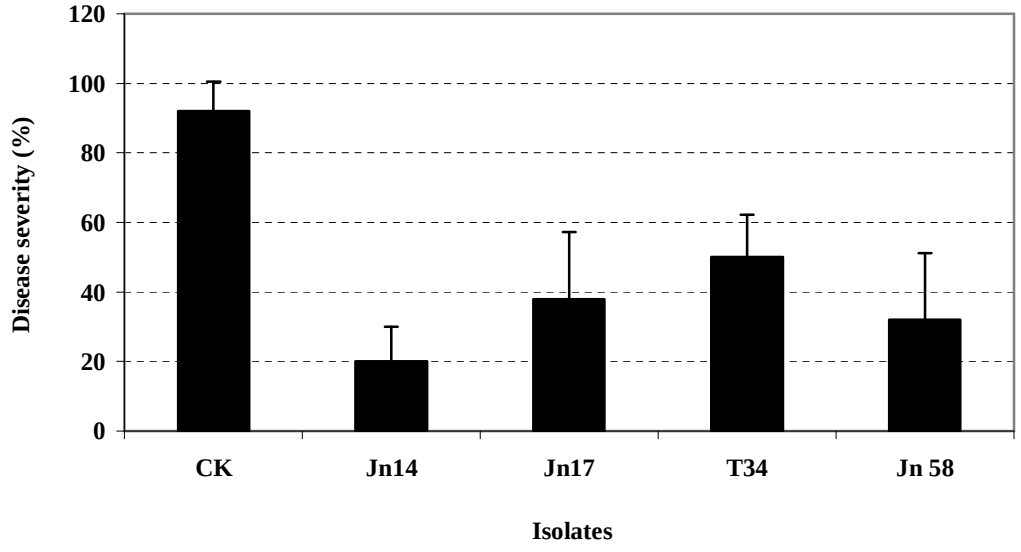


Figure (3.11) Batch III: Effect of the most promising *Trichoderma* spp. isolates on early blight (*A. solani*) disease severity (%) after 15 days incubated at 25 ºC. (LSD =19.2, P ≤ 0.05)

3.3.2 *Trichoderma* isolate's identification

According to the taxonomic keys used (Bisset, 1984, 1991 a, b, c), the isolates (Jn58 and T34) were taxonomicaly identified according to their conidial morphology, color and texture, and growth characteristics. The two isolates, identified as *T. harzianum* belonged to the section *Pachybasium* which is characterized by broad or inflated conidiophores elements and phialides, which give the conidiophores a stout or rigid appearance. Phialides were ampulliform, divergent, and arranged in crowds on terminal branches of conidiophore that at rerepeatedly branched and rebranched at an indefinite number of levels. In addition, many species have conspicuous, sterile elongation of the conidiophore main axis. However the isolates (Jn14 and Jn17) were previously identified by (Barakat *et al.,* 2006). The isolate (Jn14), identified as *T. harzianum* belonged to the section *Pachybasium* and the isolate (Jn17), identified as *T. atroviride*, has relatively large, ellipsoidal conidia and a very sparse branching system with curved or sinuous conidiophores branches and phialides.

3.3.3 Formulation studies (Spore suspension and Talc formula)

The *T. harzianum* isolates Jn14 and Jn58 were applied as spore suspension and talc formulation. Disease reduction was significant ($P \leq 0.05$) in both methods at the concentrations of ($10^7$and 10^8CFU/ml). However, there was a significant variation in disease reduction between the different treatments. Disease reduction ranged from 40 % to 58% for *T. harzianum* (Jn14), and from 36 % to 54 % for *T. harzianum* (Jn58). The highest disease reduction was recorded by the talc formulation (58%) at the concentration of (10^8 CFU/ml) by *T. harzianum* (Jn14), and (54%) at the concentration of 10^8 CFU/ml by *T. harzianum* (Jn58) as shown in (Table 3.2 and 3.3).

Table (3.2): Effect of different concentrations (CFU/ml) of *T. harzianum* (Jn14) applied in talc – formulation and spore suspension on early blight disease severity (%) caused by *A. solani* on tomato plants incubated at 25°C.

Treatments (CFU/ml)	Jn14	
	Talc	Spore suspension
CK.	80±3.5 a	80±6.1 a
10^7	29±4.2c	40±5 b
10^8	22±5.7d	32±4.5c

*Mean of five replicates ± standard deviation.

*Means followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =6).

Table (3.3): Effect of different concentrations (CFU/ml) of *T. harzianum* (Jn58) applied in talc – formulation and spore suspension on early blight disease severity (%) caused by *A. solani* on tomato plants incubated at 25°C.

Treatments (CFU/ml)	Jn58	
	Talc	Spore suspension
CK.	80±3.5 a	80±6.1 a
10^7	33±2.7c	44±4.2b
10^8	26±5.7d	35±7.1c

*Mean of five replicates ± standard deviation.

*Means followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =7).

3.3.4 Bioagent's time of application

The *T. harzianum* isolate (Jn14) was applied at different timings. Disease reduction was significant at all different timings compared to the control. The best values of disease reduction were obtained when the bioagent (*Trichoderma harzianum*, Jn14) was introduced 24 hours before the pathogen inoculation (B24). When the bioagent was introduced at the same time of pathogen inoculation (0 time), a disease reduction of 20% was obtained. However, when the bioagent was introduced 24 hours of the pathogen inoculation (A24), disease reduction obtained was 65%.

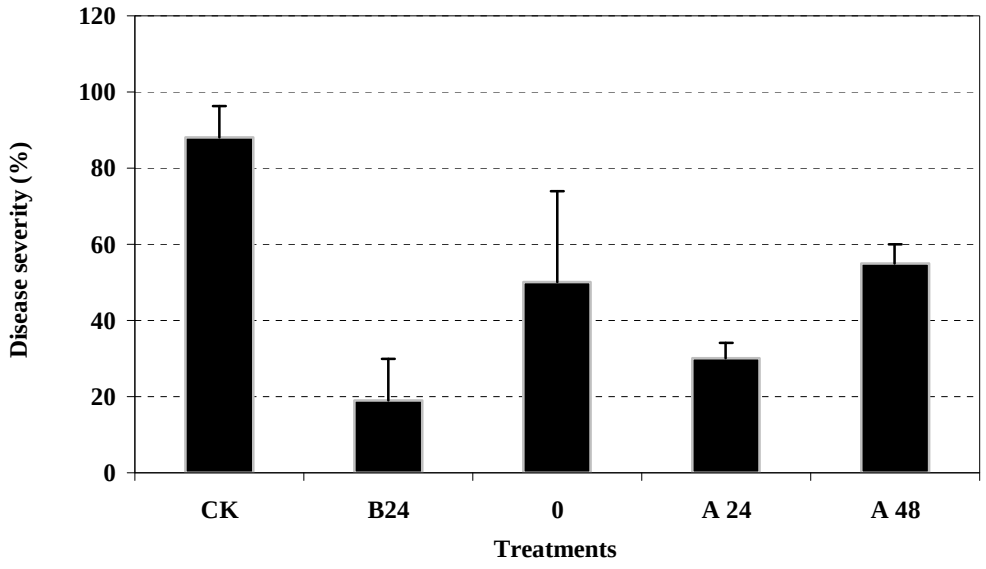


Figure (3.12): Effect of application time of *T. harzianum* (Jn14) on early blight disease severity (%). (LSD =18, P ≤ 0.05)

*(B24**):** Plants treated with *Trichoderma*, 24 hours before inoculation with *A. solani*, (A24): Plants treated with *Trichoderma*, 24 hours after inoculation with *A. solani*, (0): Plants treated with *Trichoderma and A. solani* at the same time and (A48): Plants treated with *Trichoderma* 48 hours after inoculation with *A. solani*.

3.3.5 Bioagent's inoculum concentration

The *T. harzianum* isolates, Jn14 and Jn58 were applied at different concentrations. Disease reduction was significant at all concentrations compared to the control. Disease severity decreased as inoculum concentration increased in both *T. harzianum* isolates. Disease severity was reduced by (65% and 71%) using inoculum concentrations of 10^7 and 10^8 of the isolate Jn14, respectively, (Table 3.4) compared to the control. Furthermore, disease severity was reduced by (50% and 60%) using inoculum concentrations of 10^7 and 10^8 of the isolate Jn58, respectively (Table 3.5).

Table (3.4): Effect of different concentrations (CFU/mL) of *T. harzianum* (Jn14) on early blight disease severity (%) caused by *A. solani* on tomato plants incubated at 25°C.

Concentrations (CFU/ml)	**Disease severity (%)**
CK	90±3.5a
10^5	50±3.5b
10^6	45±6.1b
10^7	25±5 c
10^8	19±2.2d

*Mean of five replicates followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =6).

Table (3.5): Effect of different concentrations (CFU/ml) of *T. harzianum* (Jn58) on early blight disease severity (%) caused by *A. solani* on tomato plants incubated at 25°C.

Concentrations (CFU/ml)	Disease severity (%)
CK	90±3.5a
10^5	60±9.4b
10^6	56±9.6b
10^7	40±9.4c
10^8	30±8.6d

*Mean of five replicates followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =11).

3.3.6 Bioagent's mode of action

The effect of fungitoxic metabolites produced by the isolates (Jn14, Jn17, T34 and Jn58) on mycelium growth rate of *A. solani* in amended media was evaluated (Figure 3.13). *Trichoderma* isolates reduced mycelia growth rate of *A. solani* in isolates Jn14, Jn58, Jn17 and T34, respectively in different rates. The variations between isolates were not statistically significant in general. *A. solani* mycelial growth reduction percentages were 97%, 95%, 83% and 72% when grown on PDA medium amended with 25% (v/v) of PDB containing metabolites produced by the isolates Jn14, Jn58, Jn17 and T34, respectively. *T. harzianum* (Jn14) recorded the highest reduction in *A. solani* mycelial growth (97%) at the concentration of 25% (v/v).

The effect of fungitoxic metabolites produced by the isolates Jn14 and Jn58 on spore germination of *A. solani* in amended media was evaluated as well (Figure 3.14). *A. solani* spore's germination reduction percentages were 32% and 30% at 25% (v/v) when grown on PDA medium amended with 25% of PDB containing metabolites produced by the isolates Jn14 and Jn58,

respectively. *T. harzianum* (Jn14) recorded the highest reduction in *A. solani* spore germination (68%) at the concentration of 25% (v/v).

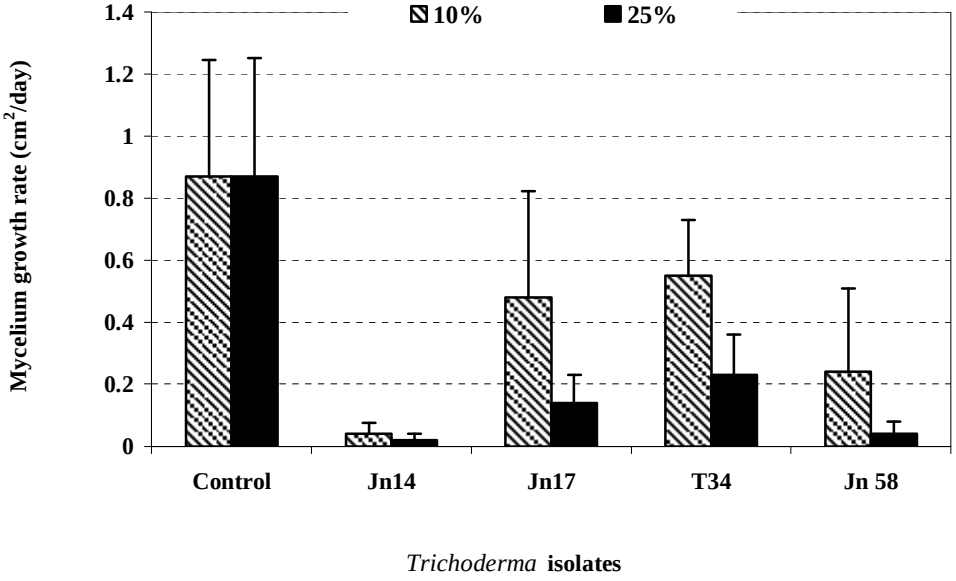


Figure (3.13): Effect *T. harzianu*m fungitoxic metabolites on mycelium growth of *A. solani* growing on PDA at 25 °C.

(LSD =0.4 P ≤ 0.05)

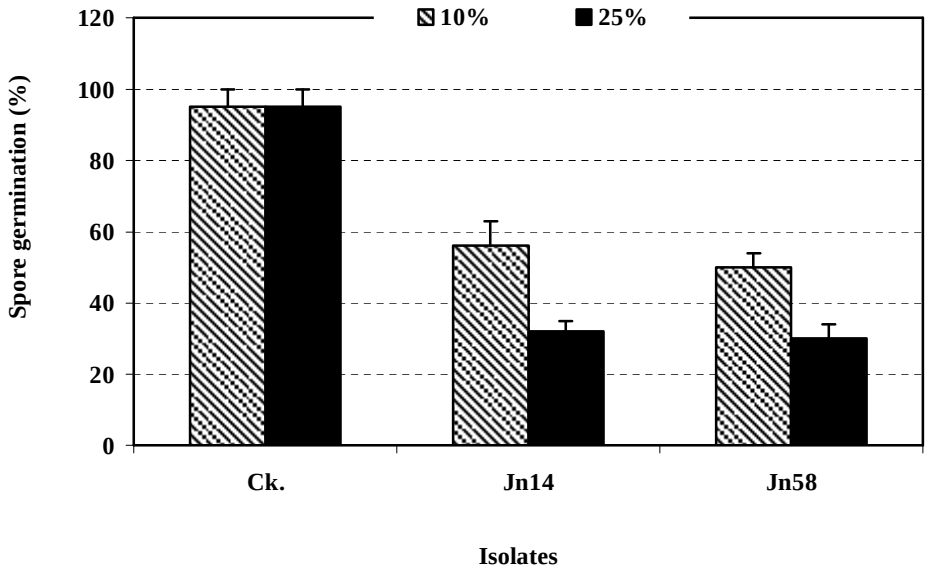


Figure (3.14): Effect *T. harzianum* fungitoxic metabolites on spore germination of *A. solani* growing on PDA at 25°C. (LSD =6, P ≤ 0.05).

3.4 Chemical control studies

3.4.1 Effect of fungicides on *Alternaria solani* mycelial growth

Preliminary results (Table 3.6) the fungicides, Propineb, Iprodione, Chlorothalonil, Maneb and Mancozeb were effective in reducing mycelium growth rate of *A. solani* at the concentrations (0.5, 1, 3, 5 and 7µg ml^{-1}). The lowest growth rates of *A. solani* mycelium were recorded by the fungicide Iprodione at all concentrations used (0.5-7 µg ml^{-1}). Iprodione inhibited mycelial growth completely. Chlorothalonil and Mancozeb came second especially at high concentrations used (5-7 µg ml^{-1}). The fungicides Propineb and Maneb recorded the lowest effect with no significant differences between various concentrations.

Table (3. 6): Effect of different concentrations of fungicides on mycelium growth rate (cm^2/day) of *A. solani* growing on PDA and incubated at 25°C.

concentrations	Propineb	Maneb	Mancozeb	Chlorothalonil	Iprodione
0	3±0.6 a	3±0.6 a	3±0.6 a	3±0.6 a	3±0.6a
0.5ppm	1.3±2.5b	1.4±2.7b	1.1±2.5b	0.8±0.5b	0±0c
1ppm	0.8±0.6b	0.8±0.3b	0.7±0.2b	0.6±0.3b	0±0c
3ppm	0.8±0.7b	0.8±0.1b	0.7±0.1b	0.6±0.2b	0±0c
5ppm	0.6±0.2b	0.6±0.1b	0.6±0.2b	0.4±0.2c	0±0c
7ppm	0.6±0.1b	0.6±0.3b	0.5±0.3c	0.4±0.2c	0±0c

*Mean of five replicates ± standard deviation

*Means followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =1.2).

3.4. 2 Effect of fungicides on *Alternaria solani* spore's germination

The fungicides, Propineb and Iprodione effect on spore's germination of *A. solani* at the concentrations of (0.5, 1 and 3 µg ml^{-1}) was evaluated

(Figure 3.15). Iprodione reduced germination at all concentrations, while Propineb was effective at higher concentrations of (1 and 3 µg ml^{-1}). There was no significant difference, however, between the two fungicides at the low concentration of (0.5 µg ml^{-1}). Spore's germination was reduced by 31% and 43 % at the concentration of (3 µg ml^{-1}) when Propineb and Iprodione were applied, respectively compared with the control.

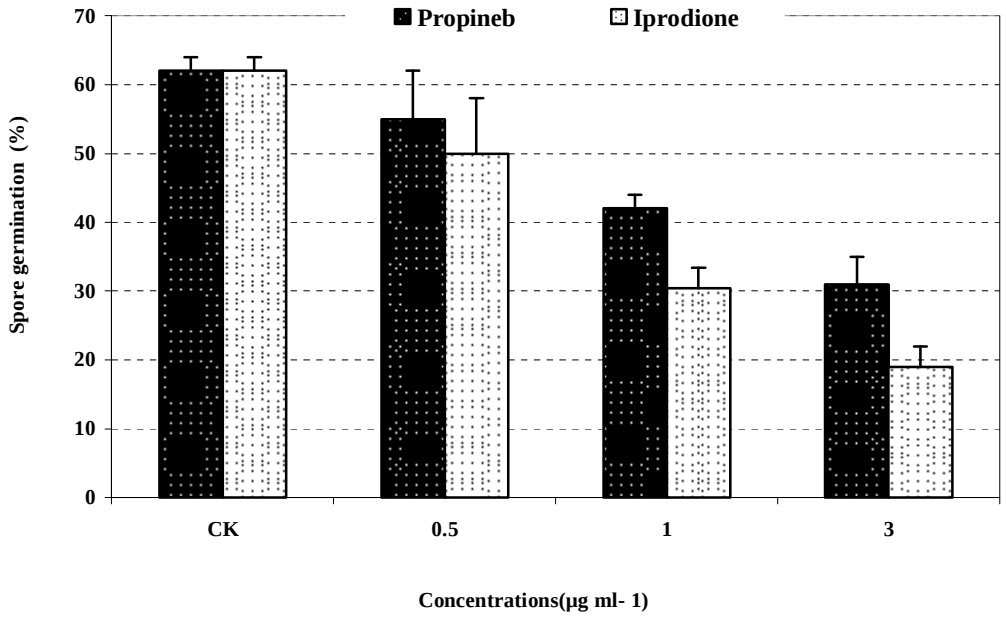


Figure (3.15): Effect of different concentrations of the fungicides Propineb and Iprodione on *A. solani* spore's germination incubated at 25°C after 3 days. (LSD =6, P≤ 0.05)

3.4. 3 Effect of fungicides on *A. solani* disease severity

The results showed that the fungicides, Propineb, Iprodione, Chlorothalonil, Maneb and Mancozeb significantly reduced early blight disease severity (%) on tomato plants grown in pots at the concentrations (1 and 3µg ml^{-1}). Iprodione recorded the highest disease reduction at both

concentrations used (Figure 3.16).

The fungicides Iprodione, Propineb, Chlorothalonil, Maneb and Mancozeb at the concentration (3µg ml^{-1}), significantly reduced disease severity by 76%, 56%, 52%, 44% and 40%, respectively, compared to the control.

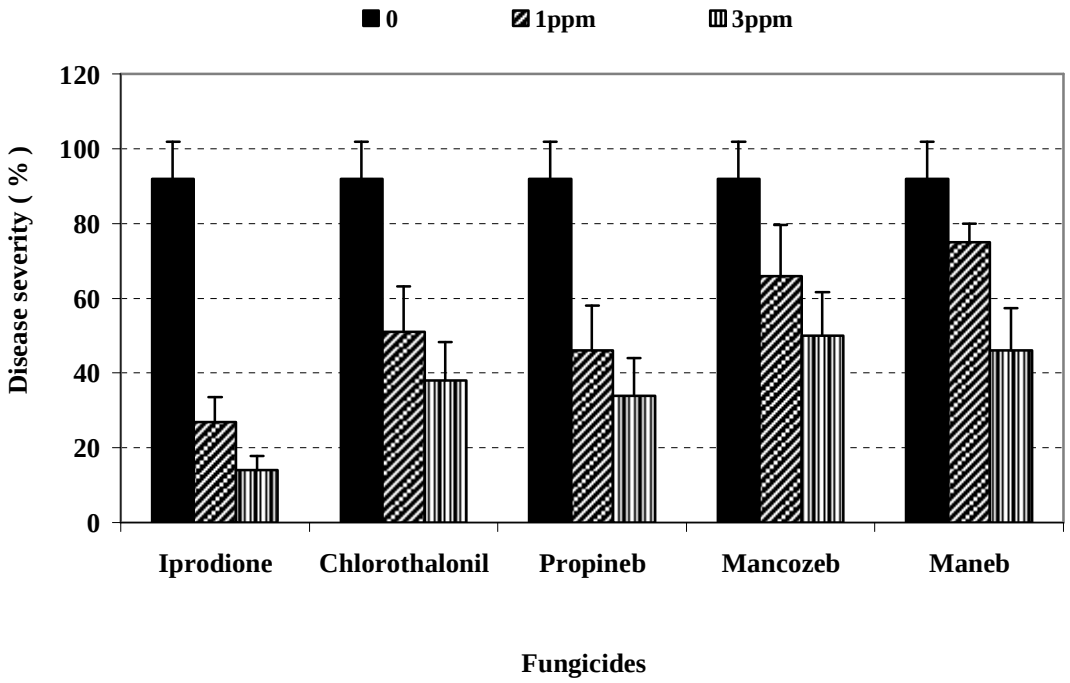


Figure (3.16): Effect of different concentrations of fungicides on *A. solani* of tomato plants incubated under growth chamber at 25°C.
(LSD =12, P≤ 0.05)

3.4.4 Effect of fungicides on *Trichoderma harzianum* spore's germination

The two fungicides, Propineb and Iprodione reduced spore's germination of *T. harzianum* isolates (Jn14 and Jn58) at the concentrations (0.5, 1 and 3µg ml^{-1}). There were significant differences between both fungicides on the rate of spore's germination at all concentrations. Iprodione was more effective in reducing germination than Propineb at all concentrations and against both isolates.

Iprodione reduced *T. harzianum* spore's germination by 47 % and 43 %

at the concentration of (3µg ml^{-1}) compared to the control for the isolates Jn14 and Jn58, respectively. Fungicides effect was almost similar for both isolates (Figure 3.17).

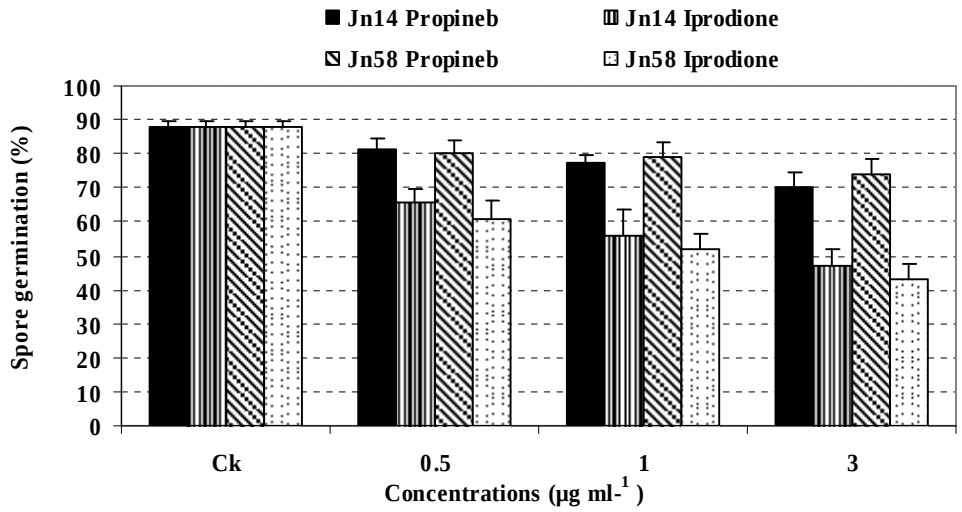


Figure (3.17): Effect of fungicides on *T. harzianum* (Jn14 and Jn58) spore's germination incubated at 25°C.

(LSD = 5, P ≤ 0.05)

3.5 Integrated control studies

3.5.1 *In vivo* studies

In this experiment the effect of combination of *T. harzianum* (Jn14) and fungicides (Iprodione, Propineb) at the concentration of (3µg ml^{-1}) on early blight disease severity was studied. All treatments reduced disease significantly in comparison with the control. The effect of *T. harzianum* was similar to the effect of the fungicide (Propineb). The highest reduction (56%) was recorded by Iprodione. *T. harzianum* combination with the fungicide (Iprodione) also registered a significant reduction of disease compared with the control.

Table (3.7): Effect of combination of fungicides and *T. harzianum* (Jn14) on early blight disease severity (%) on tomato plants grown *in vivo* (25°C).

Treatments	Disease severity (%)
Ck.	88a
Trichoderma + Propineb	73b
Trichoderma	53c
Propineb	50c
Trichoderma + Iprodione	48c
Iprodione	32d

*Mean of five replicates; values followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =5.3).

3.5.2 Field application

In this experiment the effect of combination of *T. harzianum* (Jn14) and the fungicides (Iprodione, Propineb) at the concentration of 3 µg ml^{-1} on early blight disease severity was studied (Table 3.8). All treatments reduced the disease significantly in comparison with the control. In general, field results were similar to *in vivo* assays results. Fungicides registered the highest disease reduction. Iprodine recorded the lowest disease severity values as before. Adding *Trichoderma* to fungicides was better than using *Trichoderma* alone in reducing disease severity.

Table (3.8): Effect of combination of fungicides and *T. harzianum* (Jn14) on early blight disease severity (%) on tomato plants grown in the field.

Treatments	**Disease severity (%)**
Ck.	83a
Trichoderma	68b
Trichoderma + Propineb	57c
Trichoderma+Iprodione	52c
Propineb	45d
Iprodione	35e

*Mean of eight replicates; values followed by the same letters within the same column are not significantly different according to Fisher LSD method (LSD =7).

Chapter Four

Discussion

Trichoderma species are among the most common saprophytic fungi. *Trichoderma* spp. are easily isolated from soil, decaying wood and other forms of plant organic matter (Howell, 2003).

This study demonstrated the presence of twenty six isolates of *Trichoderma* species which were recovered from 120 soil samples collected from different locations in the West Bank, during the summer of 2004. All isolates were recovered from irrigated open fields cultivated with different crops (Tomato, cucumber, bean, eggplant, pepper, squash and cabbage). Most of the isolates were recoverd from clay soil, north of the West Bank. Similiarly, research showed that *Trichoderma* spp. are generally found in all soils including forest humus layer (Wardle *et al.*, 1993) as well as in agricultural and orchard soils (Chet, 1987; Roiger *et al.*, 1991).

A successful bioagent should possess aggressive mycelial growth and high rate of spore's production. A clear variation in mycelial growth and level of sporulation was observed within *Trichoderma* spp. isolates. The highest mycelial growth and sporulation rates were recorded by the isolates (Jn14, Jn58, T34, T36, Jn17 and B44). *T. harzianum* (Jn14) obviously showed the highest mycelial growth rate (24.1 cm^2 /day), while *T. harzianum* (Jn58) recorded the highest rate of spore's production ($9.6x10^8$ spore /cm^2). This variation is very common in *Trichoderma* and has been documented by several investigators (Gullino and Garibaldi, 1994; Elad *et al.*, 1980; Henis and Chet, 1975; and Hander *et al*., 1979).

In addition, the antagonistic ability of *Trichoderma* isolates to reduce disease is highly variable (Chet *et al.*, 1979). In this study, the bioassays showed that the *Trichoderma* isolates potential in controlling *A. solani*, was highly variable as well. Disease reduction ranged between 40% and 70%. The

most effective *Trichoderma* spp. isolates were (Jn14, Jn58, Jn17 and T34). These isolates were able to reduce early blight disease severity (%) of *A. solani* on tomato plants by 70%, 58%, 52% and 40%, respectively. The most promising *Trichoderma* spp. isolate in controlling *A. solani* was the isolate Jn14. The *T. harzianum* (Jn14) was previously shown to control *B. cinerea* on tomato and bean plants (Barakat *et al.*, 2006). In addition, the same isolate was able to control several soilborne plant pathogenic fungi (*Rhizoctonia solani* and *Sclerotium rolfsii*) on tomato and bean plants (Barakat and Al-Masri, 2005). Similarly, Gullino and Garibaldi (1994) showed the reduction of tomato leaf blight disease caused by *A. solani* when *Trichoderma* spp. was included in the integrated management of the disease. Six species of *Trichoderma* including *T. harzianum, T. viride, T. koningii, T. hamatum, T. longibrachiatum and T. pseudokoningii* were tested. These antagonists were tested against the mycelia growth of the pathogen and disease incidence on tomato plants. Among the six species tested, *T. harzianum* exerted the highest inhibition of mycelial growth; disease was reduced by (50.2%). *T.viride* was next (40.9%), and then comes *T.hamatum, T.koningii, T.longibrachiatum and T. pseudokoningii*, respectively. There were no significant differences, however, among the *Trichoderma* spp. in reducing leaf blight disease. De Meyer *et al.*, (1998) found that in tomato, pepper and bean, *T. harzianum* (T39) application at sites spatially separated from the *B. cinerea* inoculation resulted in a 25–100% reduction of grey mould symptoms.

The effect of temperature on mycelium growth rate and spore production was studied for the most effective *Trichoderma* isolates. The results showed that mycelium growth rate and spore's production reached a peak at 25°C, and was completely inhibited at 35°C. Light stimulated the sporulation of *Trichoderma* isolates. The positive effect of light on spore production was also, observed by Prasun and Kanthadai (1997). They further found, that the optimum temperature for *Trichoderma* spp. growth was 25-

30°C. In addition, Chet (1990) reported that the optimal temperature for *Trichoderma* growth was around 28°C and growth was very slow below 18°C. Prasun and Kanthdai (1997) further showed that *Trichoderma* overgrew *S. rolfsii* best at 25°C and 30°C in dual culture.

In the formulation study, the most effective *T. harzianum* isolates (Jn14 and Jn58) were applied as spore suspension and talc formulation. The decrease in disease was significant in both methods at the concentrations of 10^7 and 10^8 CFU/ml on tomato plants. Talc formulation was most effective for *T. harzianum* isolate (Jn14) at the concentration of 10^8CFU/ml. Talc formulation in *Trichoderma* has been documented by several investigators (Debroah, 2002; Elad *et al*. 1999; Elad, 2000 a, b; Elad *et al*., 1995 and Elad *et al*., 1993). *Trichoderma* spores has been commercially formulated under different names (Trichodex®, wettable powder; RootPro®**,** spores mixed with peat; and Rootshield®; granules and wettable powder**).**

Similarly, Elad *et al*. (1999) and Elad (2000 a,b) who found that *T. harzianum* (T39), the active ingredient of the commercial preparation Trichodex®, has been used for the control of foliar diseases such as grey mould in various crops, and of *Pseudoperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* in cucumber and *Cladosporium fulvum* in tomato under commercial greenhouse conditions.

Applying *T. harzianum* (Jn14) 24 hours before inculation with *A. solani* gave the highest disease reduction values which gives biocontrol a protective value in this case. In addition, disease severity was negatively correlated with the bioagent inoculum concentration applied; the best concentration was 10^8 CFU/ml. There are, however, no studies conducted on time of application of the bioagent.

The mode of action of *T. harzianum* on pathogen includes mycoparasitism, competition and antibiosis (Elad, 1996). Mycoparasites utilize fungal cell-wall-degrading enzymes such as chitinases and glucanases

to dissolve their fungal hosts' cell walls and penetrate the cells (Elad, 1995). Competition is efective when the pathogen conidia need exogenous nutrients for germination and germ-tube elongation (Blakeman, 1993). Micro-organisms that produce antibiotics can also affect pathogens (Andrews, 1985), as in the case of the control of *B. cinerea* by *Bacillus* spp., (Leifert *et al.,* 1995; Seddon and Schmitt, 1999). Synergism between diferent forms of antagonism may occur, as in the case of the *in vitro* inhibition of conidial germination of *B. cinerea* by antibiotics combined with the action of cell-wall-degrading enzymes (Lorito *et al.,* 1993a, b). In this study, mycelia growth rate of *A. solani* was significantly reduced due to the production of fungitoxic substances by the *Trichoderma* isolates (Jn14, Jn58, Jn17 and T43) in liquid culture at the incubation temperature of 25°C. *T. harzianum* isolate (Jn14) was the most effective isolate against *A. solani*; it reduced the pathogen mycelium growth rate by (72%) at the concentration of 25% (v/v). At the same concentration, fungitoxic metabolites, significantly, reduced spore's germination of *A. solani* compared with the control at the same temperature. *T. harzianum* (Jn14) recorded the highest reduction in spore germination (68%). Similar results were observed by Prasun and Kanthadai (1997) who reported that *Trichoderma* spp. (isolate Td-1) produced higher concentration of fungitoxic metabolites at higher temperatures and effectively suppressed the growth of *S. rolfsii* at or below 33°C. Antibiotics have long been suggested to be involved in biocontrol by *Trichoderma* (Weindling, 1932). Sivasithamparam and Ghisalberti (1998) listed 43 substances produced by *Trichoderma* spp. that have antibiotic activity (enzymes are not included). Of these, alkyl pyrones, isonitriles, polyketides, peptaibols, diketopiperazines, sesquiterpenes, and steroids have frequently been associated with biocontrol activity of some species and strains of *Trichoderma* (Howell, 1998).

Concerning chemical control, the five fungicides, (Propineb, Iprodione,

Maneb, Chlorothalonil and Mancozeb), significantly reduced the pathogen *A. solani* mycelium growth rate at the concentrations of (0.5, 1, 3, 5 and 7µg ml^{-1}), in different rates. The highest effect on growth reduction was recorded by the fungicide Iprodione at all concentrations (0.5- 7µg ml^{-1}), and Chlorothalonil at concentrations > 3µg ml^{-1}. In addition, Iprodione reduced the pathogen spore's germination by (12%, 32% and 43%) at the concentrations of (0.5, 1 and 3, µg ml^{-1}), respectively compared with the control. Similar results were obtained by Choulwar and Datar (1989) who assessed the efficacy of the fungicides (Mancozeb and Iprodione) in reducing mycelial growth of *A. solani in vitro*; Mancozeb was more effective. Furthermore, Fadl *et al.*, (1985) *in vitro* fungicides assays showed that Ridomil was most inhibitory to linear growth of early blight of tomato at low concentration (0.5µg ml^{-1}) followed by Trimiltox Fort and Bravo 500. In bioassays, the fungicides, Propineb, Iprodione, Chlorothalonil, Maneb and Mancozeb significantly reduced early blight disease severity (%) of tomato plants grown in pots at the concentrations of (1 and 3 µg ml^{-1}). The most effective three fungicides (Iprodion, Chlorothalonil and Propineb) reduced the disease severity by 76 %, 56% and 52%, respectively, compared to the control at the concentration of (3 µg ml^{-1}). Zhang *et al.*, (2003) tested the effect of Maneb and Propineb on *A. solani*. They showed that these fungicides provided a protective mechanism but not curative.

The two fungicides, Propineb and Iprodione reduced spore's germination of *T. harzianum* isolates (Jn14 and Jn58) at the concentrations (0.5, 1 and 3µg ml^{-1}). Propineb reduced *T. harzianum* (Jn14 and Jn58) spore's germination by 20% - 25% at the concentration of (3µg ml^{-1}) compared to the control. The effect of fungicides on the *Trichoderma* isolates was less than their effect on the disease or the pathogen. This will allow using lower concentrations of fungicides with biocontrol in an integrated context. Similar results were obtained by (Sesan and Oprea, 1999) who found that fungicides

mancozeb, benomyl and vinclozolin can be used at low concentration in combination with *Trichoderma* for controlling *Sclerotium cepivorum* on onions.

In terms of integrated control, the combination of *T. harzianum* (Jn14) and the fungicides (Iprodione and Propineb) at the concentration of (3µg ml^{-1}) reduced early blight disease on plants in pots by 40% and 15% respectively, compared to the control.

In field application, the combination of *T. harzianum* (Jn14) at the concentration of 10^8 CFU/ml and the fungicides (Iprodione, Propineb) at the concentration of (3µg ml^{-1}) reduced early blight disease severity in the field by 31% and 25% respectively, compared to the control. All treatments have reduced the disease significantly compared with the control. Application of *Trichoderma* with fungicides was better than using *Trichoderma* alone. The combination of *T. harzianum* with fungicides improved the level of disease control; fungicides however, were obviously better in respect to disease reduction. Similar results were observed by Elad *et al.* (1993) who found that when *T. harzianum* was sprayed on cucumber plants, the fungicides Iprodione or Vinclozolin or alternated with (Diethofencarb + Carbendazim) increased the efficiacy of control up to 90% compared with the spraying with fungicide alone. A mixture of *T. harzianum* with a dicarboximide fungicide resulted in up to 96% control of grey mould. The alternation of sprays with the biocontrol preparation and with a dicarboximide fungicide was found to be effective, thus enabling a reduction in the use of chemical sprays. Conditions favouring the ability of *T. harzianum* to control grey mould were temperature above 20°C and relative humidity between 80 and 97%. The difference in level of disease reduction might be due to the different disease (grey mold) tested in this study, difference and variation within *Trichoderma* isolates sensetivity to fungicides and variation in environmental and microclimate conditions prevailed during the experiment. Elad, (1994)

further found that the integration of biological and chemical controls has been successful in the control of (*Botrytis cinerea*). Alternation of *T. harzianum* (T39) with chemical fungicides on a weekly basis resulted in disease suppression that was as effective as that achieved by applying the fungicide alone and was more consistent than *T. harzianum* (T39) applied alone (Elad and Zimand, 1991; Elad and Zimand, 1992; O'Neill *et al*., 1996). Elad *et al.*, (1993) also showed that *Trichoderma harzianum* preparation (in the commercial form, Trichodex®), alone or in combination or alternation with fungicides, effectively controlled *Botrytis* on greenhouse cucumbers. However, in another study, Trichodex® was only effective in controlling *Botrytis* on greenhouse tomatoes in combinatiotin with Iprodione (Malathrakis and Klironomou, 1992).

Finally, the results obtained showed that the biocontrol agent *T. harzianum* can be applied with the fungicides (Iprodione and Propineb) at low concentrations easily which allows a more friendly mean of control for the environment in an integrated context.

Conclusions

The study showed that the *Trichoderma* isolates (Jn14, Jn58, Jn17 and T34) were the most effective in reducing early blight disease of tomato. These isolates were able to reduce disease severity (%) of *A.solani* on tomato plants by 70%, 58%, 52% and 40%, respectively. The optimum temperatures for mycelium growth rate and spore's germination of *Trichoderma* isolates were in the range of 25°C - 30°C; light further stimulated the sporulation.

The optimal concentration for using the most effective *Trichoderma* isolates (Jn14 and Jn58) was 10^8 CFU/ml as talc. Concerning the mode of action, antibiosis played an important role; a significant reduction in *A. solani* mycelial growth was obtained when the pathogen was grown on PDA medium amended with *Trichoderma* metabolites.

As for control, *T. harzianum* (Jn14) combination with the fungicide (Iprodione) registered a significant reduction of disease compared with the control; combination of *T. harzianum* (Jn14) with fungicides was better than using *Trichoderma* alone in reducing disease severity.

In conclusion, biocontrol of early blight disease of tomato using native isolates of *T. harzianum* can be a valid control option but further studies are necessary to shed more light on mode of action, integrated control and large-scale field applications.

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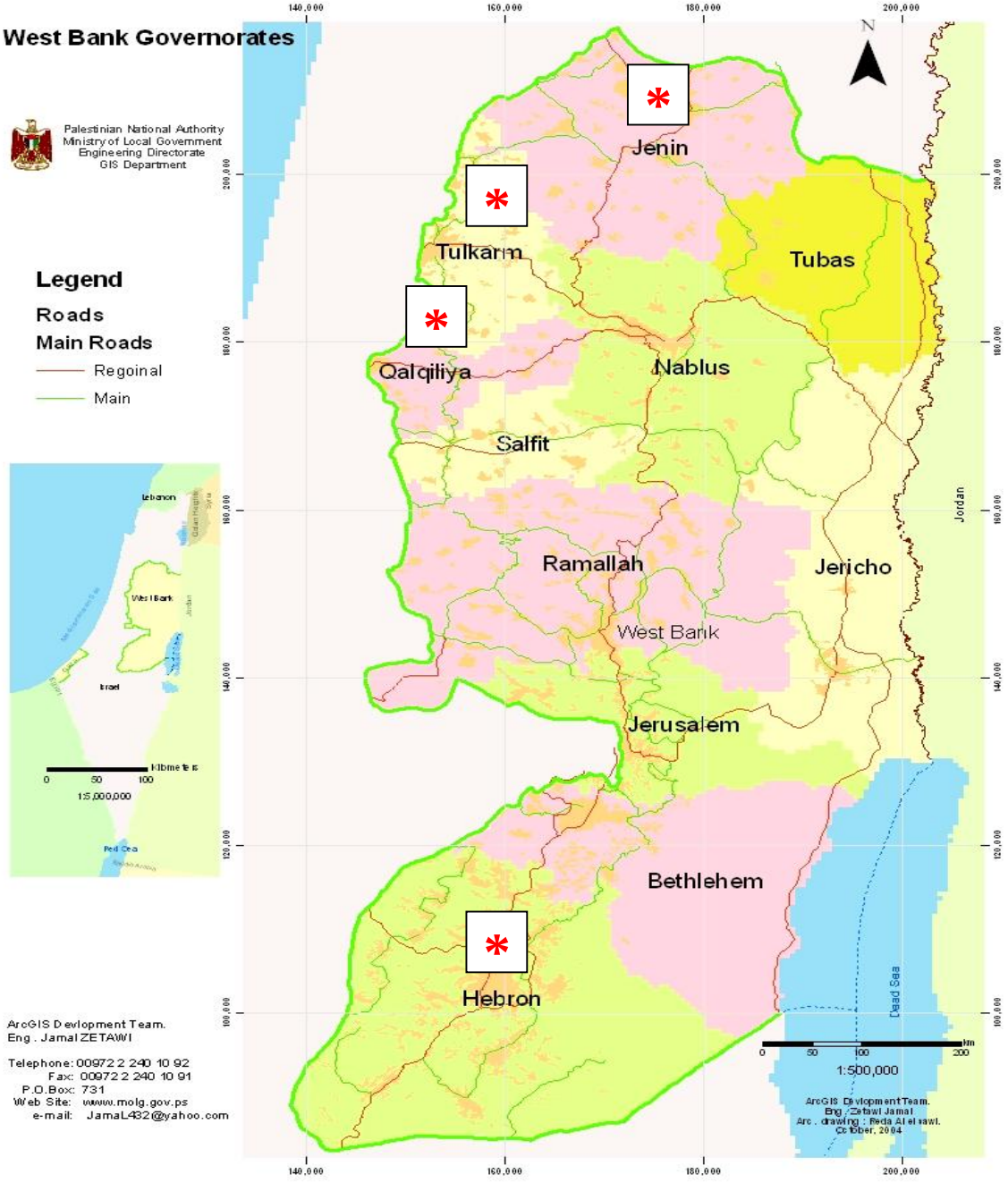
Appendices

Appendix A: West Bank map showing locations of soil sampling



Isolates were recovered from * Jenin (15) * Tulkarem (6) * Hebron (3) and (2) from Qalqelia.

Appendix B: *Trichoderma* - Selective Media (TSM) consists of the following components (gram/liter of distilled water):

Magnesium sulfate heptahydrate ($MgSO_4 .7H_2O$)	0.2g
Potassium phosphate dibasic – anhydrous (KH_2PO_4)	0.9g
Potassium chloride (KCl)	0.15g
Ammonium nitrate (NH_4NO_3)	1.0g
Glucose	3.0g
Chloramphenicol	0.25g
Dexon 60%	0.3g
Pentachloronitrobenzene (PCNB)	0.2g
Rose bengal (Tetrachlorotetradiodfluorescein)	0.15 g
Agar	20g

Appendix C:

***Alternaria solani* growing on PDA**

Conidia of *Alternaria solani* under microscope

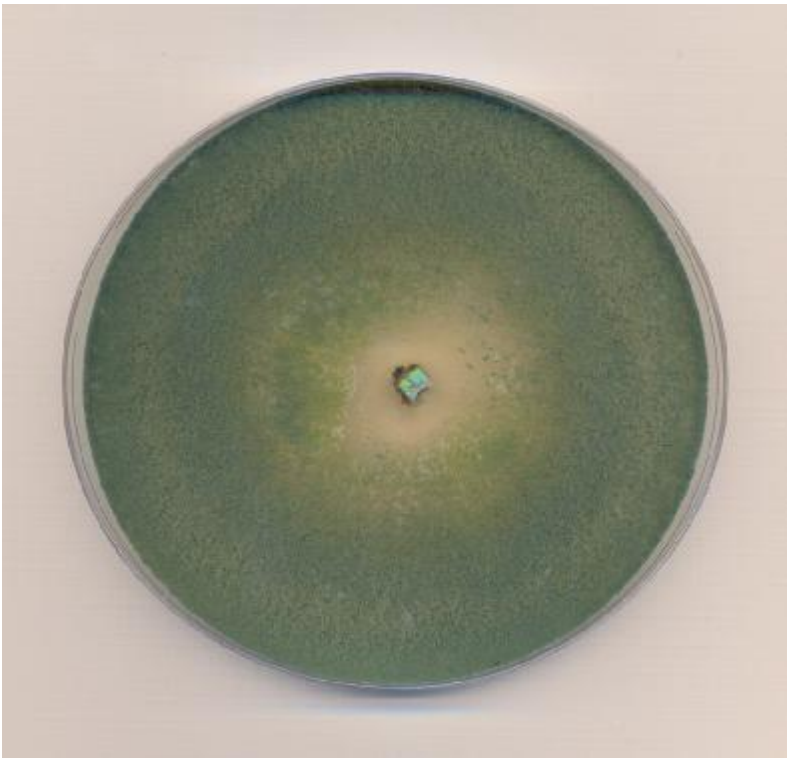
Appendix D:

Symptoms on stem

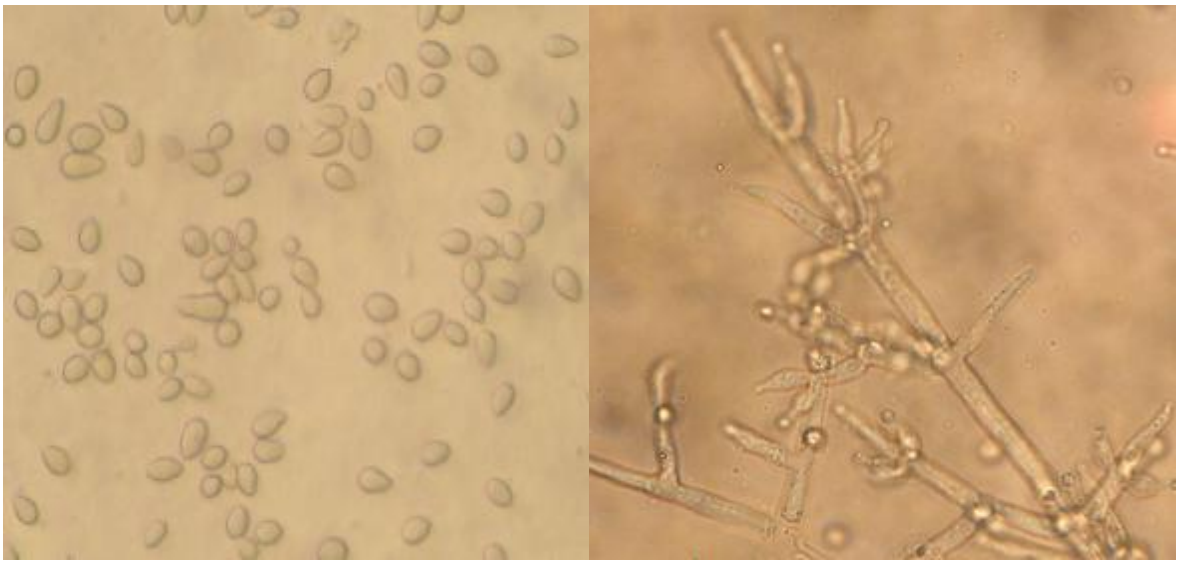
Symptoms on leaves

Symptoms on fruit

Appendix E:



***Trichoderma harzianum* (Jn14)**



Conidia and phialides of *Trichoderma harzianum* under microscope

Appendix G: ANOVA TABLES

Experiment	DF treat	DF residual	DF total	SS treat	SS residual	SS total	MS treat	MS residual	F	LSD
Mycelia growth rate (MGR) Batch I	46	141	187	4276.1	2230.6	6506.7	93	15.82	15.8	6
Mycelia growth rate (MGR) Batch II	25	52	77	1122.6	804.8	1927.4	44.9	15.5	3	6
Sporulation Batch I	46	94	140	384.9	159.4	544.3	8.4	1.7	5	2
Sporulation Batch II	25	52	77	345.8	61.8	407.6	13.8	1.19	11.6	1.8
Sporulation Batch III	25	52	77	662	110.1	772.2	26.5	2.1	12.5	2.4
The effect of T. on growth parameters (Sporulation)	39	80	119	1357.3	114.6	1472	34.8	1.4	24.3	2
The effect of T. on growth parameters (MGR)	39	160	199	29037.4	13085	42122.4	744.5	81.8	9.1	11.3
Preliminary evaluation of antagonistic potential Batch I	47	192	239	10936.3	41560.0	150956.3	2327.6	216.5	10.8	18
Preliminary evaluation of antagonistic potential Batch II	26	108	134	47638.1	19260.0	66898.1	1832.2	178.3	10.3	17
Preliminary evaluation of antagonistic potential Batch III	13	56	69	35707.1	6820.0	42527.1	2746.7	121.8	22.6	14
Preliminary evaluation of antagonistic potential Batch	4	20	24	15336.0	4240.0	1957.0	3834.0	212.0	18.1	19
Spore suspension and Talc formula (Jn14)	5	24	29	17004.1	580.0	17584.1	3400.8	24	140	6
Spore suspension and Talc formula (Jn58)	5	24	29	14170.0	630.0	14800.0	2834.0	26.3	108	6.7
Bioagent's time of application	4	21	25	14424.6	3803.3	18227.9	3606.1	181.1	19.9	17.7

Continue: Appendix G: ANOVA TABLES

Experiment	DF treat	DF residual	DF total	SS treat	SS residual	SS total	MS treat	MS residual	F	LSD
Bioagent's inoculum concentration (Jn14)	4	20	24	15614.0	370.0	15984.0	3903.5	18.5	211.0	6
Bioagent's inoculum concentration (Jn58)	4	20	24	10504.0	1420.0	11924.0	2626.0	71.0	37	11
Bioagent's mode of action (MGR)	9	20	29	2.9	1.0	4	0.32	0.052	6.2	0.4
Bioagent's mode of action (Sporulation)	4	20	24	13756.0	400.0	14156.0	3439.0	200.	1710.	6
Effect of fungicides on *Alternaria solani* mycelial growth	25	104	129	8514.4	87.5	8601.9	340.6	0.84	404.7	1.2
Effect of fungicides on *A.solani* disease severity	11	48	59	33234.6	4440.0	37674.6	3021.3	92.5	33	4.6
Effect of fungicides on *Alternaria solani* spore's germination	6	35	41	9538.9	783.7	10322.6	1589.8	22.4	71	5.5
Effect of fungicides on *T. harzianum* spore's germination	8	36	44	38704.7	569.2	39273.9	4838.0	15.8	305.9	5
Integrated control studies: *In vivo* studies	5	24	29	9936.6	400.0	10336.6	1987.3	16.6	119.2	5
Field application	5	42	47	11589.8	1903.6	13493.5	2318	45.3	51.1	6.8

المكافحة الحيوية لمرض اللفحة المبكرة على محصول البندورة باستخدام عزلات محلية من فطر التريكوديرما

الخلاصة

يعتبر مرض اللفحة المبكرة (*Alternaria solani*) من أهم الامراض الشائعة التي تصيب محاصيل العائلة الباذنجانية (بطاطا، بندورة، فلفل و باذنجان). تعتمد مكافحة المرض على استخدام العديد من الطرق وتشمل المكافحة الكيماوية، العمليات الزراعية و المكافحة الحيوية.

في هذه الدراسة تم استخدام 73 عزلة من فطر *Trichoderma* والتي تم عزلها من عينات التربة التي تم جمعها من الحقول الزراعية من مناطق الضفة الغربية المختلفة. وعند دراسة مقدرة العزلات على مكافحة المرض على البندورة أظهرت النتائج ان اقوى العزلات كانت (Jn14،Jn58 ، Jn17، T34) والتي عملت على تقليل المرض بنسبة (70، 58، 52 و 40 %) على التوالي. واوضحت الدراسة كذلك أن درجة الحرارة المثلى لنمو الفطر كانت 25 درجة مئوية وقد توقف نمو الفطر على درجة حرارة35 مئوية. كما أفادت النتائج ان استخدام اقوى العزلات الفاعلة (Jn4 و Jn58) بتركيز10^7 و 10^8 (CFU/ml) في صورة معلق ابواغ او ابواغ محمولة على مسحوق Talc ادت الى تقليل المرض بنسبة (48 % ،45%) و بنسبة (58% ، 54%) على التوالي.

وبعد فحص قدرة العزلات على انتاج المثبطات الفطرية في الوسط الغذائي (PDB) الممزوج مع الوسط الغذائي (PDA) بنسبة (25%)، أظهرت النتائج ان العزله Jn14 ثبطت نمو الفطر (*Alternaria solani*)بنسبة 72% على درجة حرارة 25 مئوية.

وعند فحص كفاءة المبيدات الفطرية (Iprodione، Chlorothalonil، Propineb، Mancozeb، Maneb) في مكافحة المرض على نباتات البندورة الكاملة تحت ظروف المختبر بتركيز (3 ميكروغرام/مل) أظهرت النتائج ان هذه المبيدات ادت الى تقليل المرض بنسبة (76%، 56%، 52%، 44%، 40%) على التوالي.

و عند استخدام اقوى عزلات فطر التريكوديرما Jn14 بالتزامن مع المبيدات الفطرية (Iprodion, Propineb) في مكافحة المرض على نباتات البندورة في الحقل المفتوح اظهرت النتائج ان استخدام فطر التريكوديرما مع المبيدين بتركيز(3 ميكروغرام/مل) عملت على تقليل المرض بنسبة (31% و 25%) على التوالي مقارنة مع الشاهد.