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**Enhancement of the biocontrol potential of *Trichoderma harzianum* in  
controlling *Fusarium oxysporum* on tomato.**

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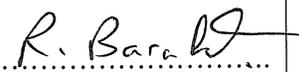
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## List of Acronyms

BCA	Biocontrol agent
CFU	Colony forming unit
CK	Control
Cm	Centimeter
CWDs	Cell wall degrading enzymes
Gm	Gram
PDA	Potato dextrose agar
PDB	Potato dextrose broth
Ppm	Part per Million
Rpm	Round per minute
SDW	Sterile distilled water
Spp	Species
<i>T. harzianum</i>	<i>Trichoderma harzianum</i>
<i>F. oxysporum</i>	<i>Fusarium oxysporum</i>
Foxy	<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i>
TSM	<i>Trichoderma</i> Selective Media
WP	Wettable powder
$\mu$ M	Micro molar
mM	Milli molar

## **Enhancement of the biocontrol potential of *Trichoderma harzianum* in controlling *Fusarium oxysporum* on tomato.**

### **Abstract**

Tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Lo7) is a serious disease in greenhouses and open fields in the temperate regions of the world. Biological control of the disease using *Trichoderma* species has been well recognized. The present study investigated the effect of exogenously applied nutrients ( $\text{NH}_4$ ,  $\text{NO}_3$ , Sugars,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{P}^+$ ) on conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp *lycopersici* (Lo7) in vitro, and in vivo. Furthermore, the increased growth response of tomato plants in the presence of the biological control agent *T. harzianum* (Jn14) was assessed. In addition to the enzymes production (Chitinases and Glucanases) by *T. harzianum* (Jn 14), and their effect on conidia germination, and germtube growth of *F. oxysporum* f.sp *lycopersici*. The results showed that  $\text{NO}_3$ ,  $\text{Ca}^{2+}$  and  $\text{P}^+$ , significantly stimulated conidia germination and germtube growth of *T. harzianum* (Jn14) at concentrations (0.01-1mM), but reduced conidia germination and germtube growth of *F. oxysporum* f. sp. *lycopersici* (Lo7) in vitro. Furthermore,  $\text{NO}_3$ ,  $\text{Ca}^{2+}$ ,  $\text{P}^+$  and  $\text{Fe}^{2+}$  significantly increased the population of *T. harzianum* (Jn14), but decreased the population of *F. oxysporum* f. sp. *lycopersici* (Lo7) in vivo. In addition, tomato plants growth parameters were stimulated in the presence of *T. harzianum* (Jn14). Concerning enzyme's production, *T. harzianum* (Jn14) produced 15% more endochitinases than *T. asperillum* (T 203), while the later produced 2% more of the exochitinases. In addition, the isolate Jn14 of *T. harzianum* produced 21% more glucanase than the isolate Jn58. Concerning antibiosis, *T. harzianum* (Jn14) culture filtrates inhibited conidia germination and germ tube growth of *F. oxysporum* f. sp. *lycopersici* (Lo7) at the concentration of 1000  $\mu\text{l/ml}$  in vitro.

# 1. INTRODUCTION

## 1.1 *Fusarium* wilt of tomato

Tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is a serious disease which causes heavy crop losses worldwide. Several management options, including using plant resistant varieties, balanced nitrogen fertilizer, four year crop rotation, soil fumigation and soil solarization have been suggested to control the disease (Ioannou *et al.*, 2000). In Palestinian agriculture, *Fusarium* wilt is a serious disease of greenhouses and open field crops ( Barakat and Al Masri, 2011). The disease management is very difficult due to its endophytic growth and persistence in soil. It has become one of the most damaging diseases wherever tomatoes are grown intensively due to the pathogen persistence in the infested soils (Soytong, 2007).

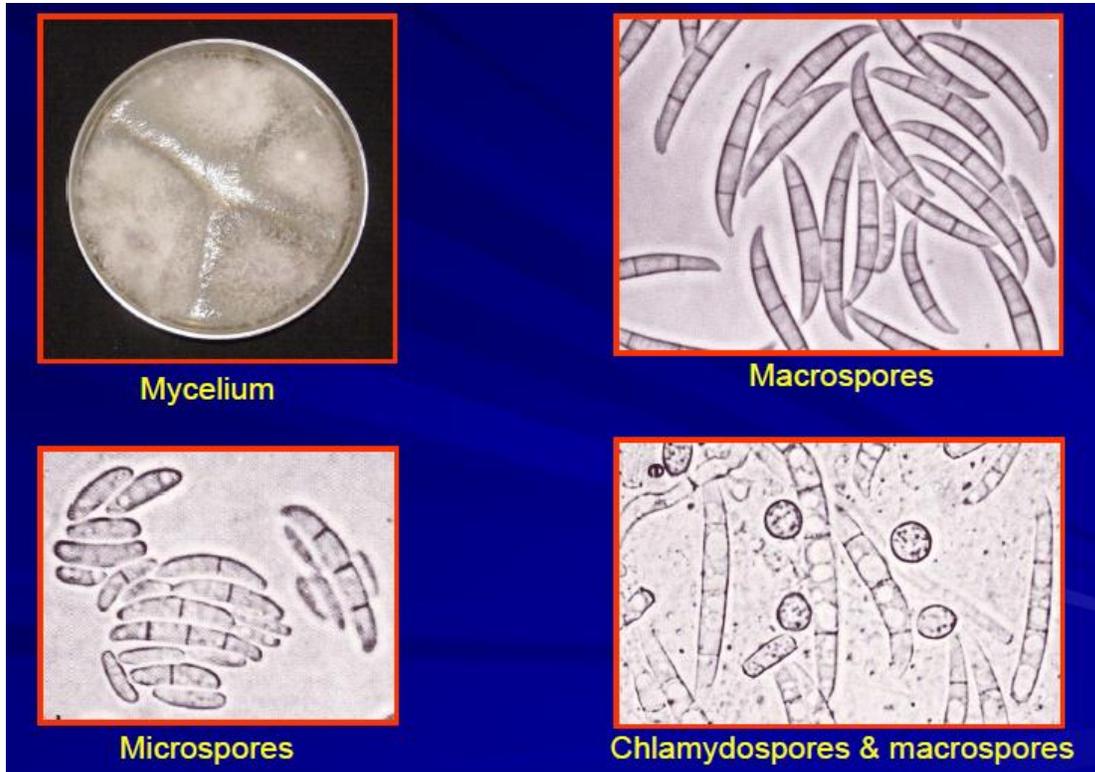
The disease was initially described by Masee in 1890; walker, 1971 and Jones and Woltz (1981). The disease occurs in at least 32 countries, and in most of tomato-growing regions of the world. The causal fungus is one of the most important genera of plant pathogenic fungi with a record of devastating various economically important plants (Messian *et al.*, 1991; Armstrong and Armstrong, 1981).

### 1.1.1 The Pathogen

*Fusarium* is a well distributed large genus of filamentous fungi affecting plant, animal and human health as they enter the food chain (Agrios 1988; Smith *et al.* 1988). They produce toxins, fumonisins and trichothecenes. *F. oxysporum* infects a wide range of hosts that include sugarcane, garden beans, cowpeas, potatoes, banana, water melon, prickly pear, tomato, cucumber, pepper, muskmelon, tobacco, cucurbits, sweet potatoes, asparagus, vanilla, strawberry and cotton (Naik *et al.* 2010; Nikam *et al.* 2011). Two formae speciales of this fungus are known to affect tomato, a crop plant of great economic importance *F. oxysporum* f.sp. *lycopersici* (FOL) which cause a severe wilt disease, whereas *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) which causes crown and root rot (Jones 1991).

*F. oxysporum* f.sp. *lycopersici* is the causative agent of vascular wilt disease in tomato. It belongs to the Mitosporic Fungi (Ascomycota) in the class *Sordariomycetes* and order Hypocreales (Agrios, 2005). As in other *Fusaria*, its identification has generally been based on morphological criteria such as the shape of micro and macroconidia, structure of microconidiophores and formation and disposition of chlamydospores (Henni *et al.*, 1994; Di Pietro *et al.*, 2003). No sexual stage has been described for *F. oxysporum*. Consequently, other mechanisms of genetic

exchange between individuals determine genetic diversity in different populations of this fungus (Ploetz and Correll, 1988; Katan *et al.*, 1991).



**Figure 1.** *Fusarium oxysporum* types of spores. (Pictures taken from Toussoun, T.A., and Nelson, P.E. 1976)

### 1.1.2 Disease cycle and epidemiology

*F. oxysporum* f. sp. *lycopersici* is a soil borne pathogen which can persist many years in the soil without a host. Most infections originate from the population associated with infected tomato debris. Healthy plants can become infected by *F. oxysporum* f. sp. *lycopersici* if the soil in which they are growing is infested with the pathogen (Mijatović *et al.*, 2007). In addition, *F. oxysporum* f. sp. *lycopersici* survives in infected plant debris in the soil as mycelium and in all its spore forms but, most commonly,

especially in the cooler temperate regions, as chlamydospores in plant debris (Agrios, 2005). The fungus can be a parasite of many weeds, seeds and several other hosts, where it colonizes the cortices of roots. The pathogen can colonize as well tomato roots and then infect the xylem, where by it moves passively through the plants vascular system. Usually, infection into the xylem is favored by wounds to the roots, including those caused by the root-knot nematodes. Secondary spread of the pathogen from one plant to another seldom occurs. However, long distance dispersal of the pathogen can occur by movement of infested soil equipment, packing boxes, and workers or infested transplants. It has also been reported that the pathogen can aerielly disseminate under certain environmental conditions. (Correll and Jones, 2014). (Figure 2).

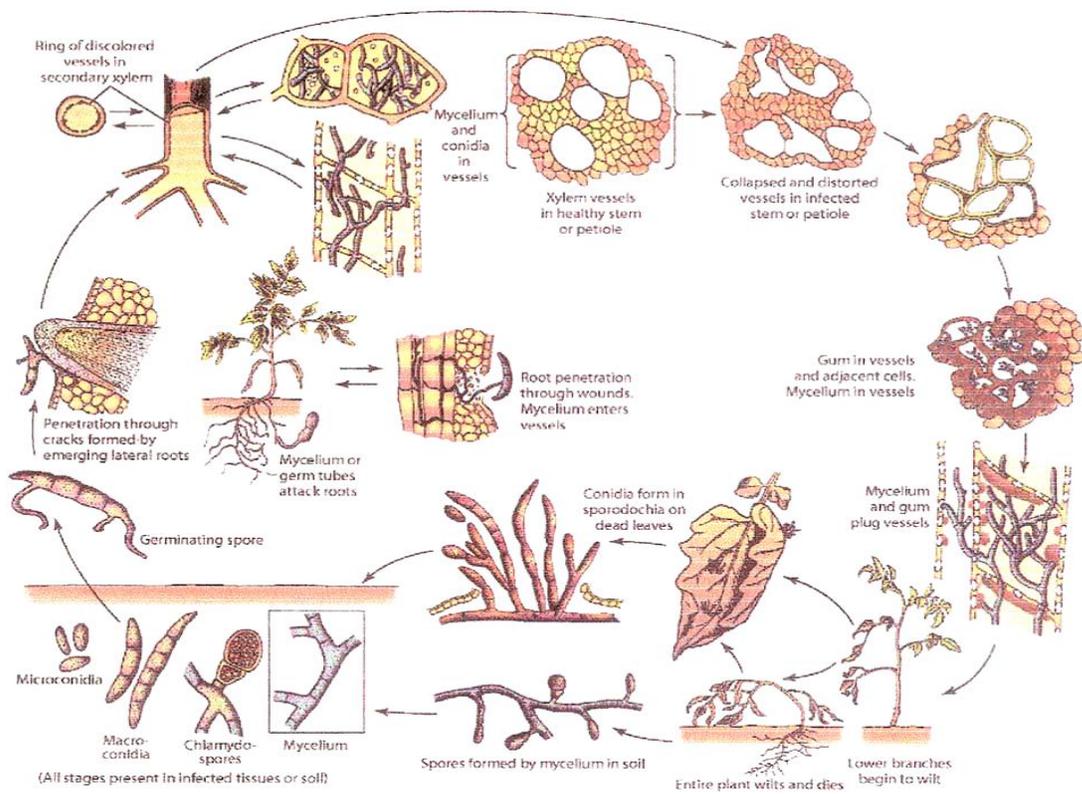


Figure 2. Disease cycle of *Fusarium oxysporum* f.sp *lycopersici*. (Agrios, 2005).

### 1.1.3 Disease Control

*Fusarium* wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* is difficult to control (Borrero *et al.*, 2006; Elmer, 2006). Numerous strategies have been proposed to control the pathogen (Biondi *et al.*, 2004; Ahmed, 2011). One strategy was the use of resistant tomato varieties which was only practical measure for controlling the disease in the field. Several such varieties are available today in the market. Adding to this fact and as the fungus is so widespread and so persistent in soils, seedbed sterilization and crop rotation, even though considered as sound practices, are of limited

value. Also, soil sterilization is too expensive so far for field application levels, but it should be however, implemented for greenhouse grown tomato plants. Use of healthy seeds and transplants is of course mandatory, and hot-water treatment of seed suspected of being infected should precede planting (Agrios, 2005).

#### **1.1.3.1 Cultural methods**

The most effective means of managing Fusarium wilt of tomato is to use disease resistant cultivars. (Correll and Jones, 2014). 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). Correll and Jones, 2014 showed that planning a 3-5 year rotation to control Fusarium wilt of tomato can be a feasible option. Limiting available nutrients is a key for general suppression. With an abundance of free nutrients, the pathogen can prosper. Virtually, any treatment to increase the total microbial activity in the soil will enhance general suppression of pathogens by increasing competition for nutrients (Granatstein, 1998).

A direct correlation between adequate calcium levels, and/or higher pH levels, and decreasing levels of Fusarium wilt occurrence has been established for tomatoes (Jones *et al.*, 1989).

Nitrate forms of nitrogen fertilizers may suppress Fusarium wilt of tomato, while the ammonia form increases disease severity. The nitrate form tends to make the root zone less acidic. Basically, the beneficial effects of high pH are lost by using acidifying ammonium nitrogen. Previous studies on tomatoes have shown that the use of nitrate nitrogen in soil with an already high pH results in even better wilt control (Woltz and Jones, 1973).

Woltz and Jones (1981) demonstrated that Fusarium wilt of tomato was reduced in low phosphate soils making the pathogen more vulnerable than the host.

In general, the combination of lime, nitrate nitrogen, and low phosphorus is effective in reducing the severity of Fusarium wilt of tomato. In conclusion, Fusarium wilt of tomato can be reduced in some soil types by using calcium nitrate fertilizers, by avoiding the use of ammonium nitrate fertilizers, and by raising the soil pH to 6.5-7.0.( Correll and Jones, 2014).

### 1.1.3.2 Disease resistance

Disease resistance against *F. oxysporum* f. sp. *lycopersici* has been investigated by several researchers (Wu *et al.*, 2005; Djordjević *et al.* 2011b). Djordjević *et al.* (2011b), evaluated tomatoes for Fusarium wilt resistance, and showed that race 1 of Fusarium wilt is not a limiting factor for successful tomato production, but race 3 of *F. oxysporum* f. sp. *lycopersici* can seriously endanger tomato production (Djordjević *et al.*, 2011b). Chemicals such as ethyl methane sulphonate and diepoxybutane induce mutations in tomato plants (Wu *et al.*, 2005). In protoplast culture, the genetic pool of plants can be widened by means of protoplast; this method is employed for the production of normal hybrid plants where sexual recombination is not possible (Marshall, 1993). Protoplast-derived tomato plants showed resistance against *F. oxysporum* f.sp. *lycopersici* (Shahin and Spivey, 1986).

### 1.1.3.3 Chemical methods

The most effective method in preventing tomato from Fusarium wilt infections is by mixing the tomato seeds with chemical fungicides. However, the use of chemical fungicides can be harmful to other living organisms besides reduction of soil microflora (Lewis *et al.*, 1996).

There is a constant threat that pathogens may become resistant to fungicide treatment. As for example, various pathogens became resistant to methyl benzimidazole (Baldwin and Rathmell, 1988). Other classes of fungicides were tested against *F. oxysporum* f.sp. *lycopersici*. The demethylation-inhibiting (DMI) fungicides (prochloraz, propiconazole and cyproconazole/propiconazole) act by inhibiting the demethylation step in the biosynthesis of sterols needed in fungal walls. Prochloraz proved to be the most effective fungicide against the Fusarium wilt pathogens of tomato (Song *et al.*, 2004; Nel *et al.*, 2007).

Apart from the use of fungicides, chemical treatment can also include the use of surface sterilants, fumigants and plant activators. Sterilants that have been used successfully against Fusarium wilt of tomato included formaldehyde, copper sulphate and copper oxychloride (Moore *et al.*, 1999). Other fumigants such as combination of 1,3-dichloropropene and chloropicrin were proposed as replacements of methyl bromide in the control of *F. oxysporum* f.sp. *lycopersici* (Gilreath and Santos, 2004). In

addition to all these chemical treatments, soil solarization has proved to reduce Fusarium wilt of tomato and provided good control of tomato wilt (Tamietti and Valentino, 2006).

Plant activators such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole-7-carbothioic acid S-methyl ester (BTH), commercially known as Bion®, are the best studied chemical elicitors available (Oostendorp *et al.*, 2001) and both are functional analogs of salicylic acid. They can elicit a systemic form of induced resistance across a broad range of plant–pathogen interactions (Vallad and Goodman, 2004). Apparently, one of the requirements of plant activators is that they do not display any antimicrobial activity (Kessmann *et al.*, 1994). For example validamycin A (VMA) and validoxylamine A (VAA) were not antifungal against *F. oxysporum* f.sp. *lycopersici* in vitro (Métraux *et al.*, 1991; Ishikawa *et al.*, 2005). Chitosan and chitin are known to be potential elicitors in plant defense responses, and have proved to stimulate chitinases and formation of wall appositions in tomato plants (Benhamou and Theriault, 1992).

#### **1.1.3.4 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.

Biological control of *F. oxysporum* f. sp. *lycopersici* causing wilt disease of tomato was studied *in vitro* as well as under pot conditions. Dual culture technique showed that *T. harzianum* inhibited the radial colony growth of the test pathogen (Alwathnani and Perveen, 2012).

*Trichoderma* spp. used alone or in combination with organic amendments against *F. oxysporum* f. sp. *lycopersici* on tomato plants has suppressed tomato wilt in contained soil and improved the efficacy of biocontrol against the pathogen (Cotxarrera *et al.*, 2002; Noble and Coventry, 2005; Spadaro and Gullino, 2005).

Suppressive soils are good sources of potential biocontrol agents. Once a putative biological control agent has been identified, it is important to identify the mechanisms whereby it controls the pathogen in order to find efficient ways to apply and manage *F. oxysporum* f.sp. *lycopersici*. The

biocontrol agent must also be safe to humans and plants so that it can be used in the field.

#### **1.1.3.4.1 Suppressive soils**

Soils where high levels of crop production can be maintained despite the presence of the pathogen, a susceptible host plant, and climatic conditions favorable for disease development are referred to as suppressive soils (Alabouvette *et al.*, 1993; Hoitink *et al.*, 1993). Soil may exert its influence through its physiochemical characteristics, its biological characteristics, or both (Alabouvette *et al.*, 1996). The physical and chemical characteristics include soil texture and structure, soil water, clay type, pH, micronutrients and organic matter (Alabouvette *et al.*, 1996). Microorganisms and their metabolites represent the biological component of suppressive soils (Alabouvette *et al.*, 1996). For instance, the fluorescent Pseudomonads produce several types of metabolites such as siderophores and antibiotics that can compete and are toxic to Fusarium wilt pathogens, respectively (Schouten *et al.*, 2004).

#### 1.1.3.4.2 Biological control agents

Root-colonizing plant-beneficial bacteria and fungi are important in protecting plants from root pathogens (Haas and Défago, 2005). The principal groups of plant-beneficial organisms controlling Fusarium wilt of tomato consist of bacterial species belonging to *Pseudomonas* and *Bacillus*, and non-pathogenic *F. oxysporum* (Fravel *et al.*, 2003; Haas and Défago, 2005).

Several other microbes have been reported to reduce Fusarium wilt of tomato incidence. These include the actinomycetes (Cao *et al.*, 2005), and fungi such as *Trichoderma spp.* (Harman *et al.*, 2004) and *Gliocladium spp.* (Sivan and Chet, 1986). Biocontrol organisms alone have the ability to reduce disease incidence, but often perform more efficiently when used in combination with other biocontrol agents and different integrated disease management strategies.

Use of environmentally friendly biological control agents can more effectively control the soilborne pathogens. (Saleem *et al.*, 2000). The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. These properties have made

*Trichoderma* an ubiquitous genus present in any habitat and at high population densities (Chet I, *et al.* 1997).

*Trichoderma* BCAs control ascomycetous, deuteromycetous and basidiomycetous fungi, which are mainly soil-borne but also airborne pathogens (Monte, 2001). *Trichoderma* is more efficient in acidic than alkaline soils. Excellent results of integrated control have been attained with strains of *T. virens* and metalaxyl against *Pythium ultimum* infecting cotton (Chet, *et al.* 1997), of *T. harzianum* and captan against *Verticillium dahliae* infecting potato (Chet I & Inbar J 1994), of *T. virens* and thiram against *Rhizoctonia solani* infecting tobacco, and others (Chet I, *et al.* 1997).

Sarker *et al.* (2013) investigated the efficacy of some antagonistic rhizosphere microorganisms against *F. oxysporum* f. sp. *lycopersici* causing Fusarium wilt of tomato. Probable 20 antagonistic bacterial isolates and one antagonistic fungal isolate (*T. harzianum*) from rhizosphere soil were screened out against *F. oxysporum* f. sp. *lycopersici*.

## 1.2 *Trichoderma* species

### 1.2.1 Biology and Taxonomy

*Trichoderma* are free-living soilborne fungi which are highly interactive in the rhizosphere and foliar environments. *Trichoderma* are known as imperfect fungi but now their perfect stage (*Hypocrea*) is known. It is a fast growing fungus in culture, and produces numerous green spores and chlamydospores. *Trichoderma* have created eco-friendly, safe and non-chemical disease management system which have great importance in organic agriculture. *Trichoderma*, a soilborne mycoparasitic fungus is effective against many soil borne phytopathogens ( [Rajkonda et al. 2011](#), [Dolatabadi et al. 2012](#)).



**Conidia**



**phialides**



**colony**

**Figure 3.** *Trichoderma harzianum* Jn14 conidia, phialides, and colony.

*Trichoderma* spp. are also considered cellulolytic ascomycetes and among the organisms responsible for the destruction of cellulosic fabrics ([Elsas et al, 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*.

Most *Trichoderma* strains have no sexual stage but instead produce only asexual spores. However, for a few strains the sexual stage is known, but not among strains that have usually been considered for biocontrol purposes. The sexual stage, when found, is within the Ascomycetes in the genus *Hypocrea*. Traditional taxonomy was based upon differences in morphology, primarily of the asexual sporulation apparatus, but more molecular approaches are now being used. Consequently, the taxa recently have gone from nine to at least thirty-three species (Yedidia, *et.al.* 1999).

**Kingdom:** Fungi

**Division:** Ascomycota

**Subdivision:** Pezizomycotina

**Class:** Sordariomycetes

**Order:** Hypocreales

**Family:** *Hypocreaceae*

**Genus:** *Trichoderma*

**Species:** *T. harzianum*

### 1.2.2 Ecology

*Trichoderma* spp. is widely distributed all over the world (Domsch *et al.*, 1980) and occurs 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 due to 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. 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. 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). In addition, *Trichoderma* can tolerate fungicides, such as methyl bromide, captan and maneb (Ruppel *et al.*, 1983).

### **1.2.3 *Trichoderma harzianum*, the bioagent**

The fungus *Trichoderma harzianum* is a biological control organism against a wide range of soil-borne pathogens and induce plant growth capacity. It has been shown that *T. harzianum* stimulated the growth of tomato plants (McGovern *et al.*, 1992; Datnoff and Pernezny, 1998). In addition, *T. harzianum* is well documented as effective biological agents for *R. solani* control in soil (Howell, 2003). Application of small non-effective doses (1-2 µg/kg) of Pentachloronitrobenzene (PCNB) to soil along with a *T. harzianum* preparation (2g/kg) decreased the incidence of eggplant damping off caused by *R. solani* from 13 to 40%, while *T. harzianum* alone reduced disease incidence by 26% (Hadar *et al.*, 1979).

Windham *et al.* (1989) reported reduced egg production in the root-knot nematode *Meloidogyne arenaria* following soil treatments with *T. harzianum* (T-12) preparations. The combination of sublethal heat treatment dose and *T. harzianum*, enhanced control of disease on beans (by 90 to 100%) under greenhouse conditions (Elad *et al.*, 1980).

Barakat and Al-Masri, (2009) studied the effect of the biocontrol agent *T. harzianum* (isolate Jn14) in combination with an amendment of sheep manure on the soil suppressiveness of Fusarium wilt of tomato over a 28-month period. They tested a combination of *T. harzianum* and organic amendment at the concentrations (w:w) of 6 and 10%; this reduced tomato wilt by 21–36 % and 29–36% respectively, after 0–28 months of soil incubation. When the amendment was added at the lower concentrations (2%), the wilt was suppressed only after 18–28 months.

Noble and Coventry (2005) showed also that the combination of *T. harzianum* and organic amendment can be used to control soilborne pathogens including *R. solani*. Increased growth response has been demonstrated by several other investigators (Altomare *et al.*, 1999). They demonstrated the ability of *T. harzianum* to solubilize insoluble tricalcium phosphate in vitro. Several other *Trichoderma* spp. were formulated as well,

in commercial production for the protection and growth enhancement of a number of crops in several countries (Mcspadden & Fravel, 2002)

#### **1.2.4 *Trichoderma* Modes 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 Pythiaceae fungi, ( $\beta$ -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). Mondéja *et al.*, (2010) reported the *T. harzianum* isolates T-30 and T-78 showed the greatest mycoparasitic potential against *F. oxysporum*, which could lead to improved biocontrol of this phytopathogen.

#### **1.2.4.2 Antibiosis**

Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* spp. that inhibit the growth of other microorganisms (Vey *et al.*, 2001). Most *Trichoderma* spp. 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-pentyl- $\alpha$ -pyrone, massoialactone, 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).

#### **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.*, 1997). 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 *et al.*, 2001). *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  $\beta$ -1, 3-glucanases. In addition,  $\beta$  -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, thereby 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 thereby destroy their capacity to act on plant cells.

### 1.3 Study Objectives

This study was designed and implemented to achieve the following objectives:

1. Characterize the effect of the exogenously applied cations ( $\text{NH}_4$ ,  $\text{NO}_3$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{P}^+$ ), and sugars (Glucose, Fructose and Sucrose) on conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp *lycopersici*. (Foxy Lo7) in vitro.
2. Investigate the effect of the exogenously applied cations ( $\text{NH}_4$ ,  $\text{NO}_3$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{F}_2^+$  and  $\text{P}^+$ ) on biocontrol efficiency of *T. harzianum* (Jn 14) against and *F. oxysporum* f.sp *lycopersici*. (Foxy Lo7) in vivo.
3. Evaluate the Increased growth response in tomato plants (IGR) induced by *T. harzianum* (Jn14).
4. Assess enzymes production (Chitinases and Glucanases) by *T. harzianum* (Jn 14), and their effect on conidial germination and germ tube elongation of *F. oxysporum* f.sp *lycopersici*.

## **2. MATERIALS AND METHODS**

### **2.1 Fungal isolates**

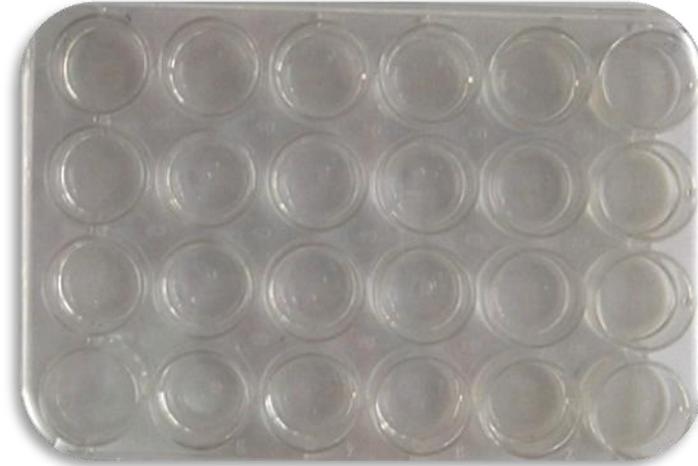
The fungal isolates of *T. harzianum* (Jn14 and Jn58), *T. asperillum* (T203), and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) used in this study were provided by the Plant Protection Research Center (PPRC) Fungal collection.

### **2.2 The effect of the exogenously applied nutrient on conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in vitro.**

#### **2.2.1 Effect of Sugars.**

The role of carbon sources in conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was investigated using three sugars: Glucose, Fructose, and Sucrose in six molar concentrations (0.001 mM, 0.01 mM, 0.1 mM, 1 mM, 10 mM, and 100 mM). Sugar solutions were prepared in distilled water and sterilized in the autoclave for 30 minutes at 127°C. *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) were grown on PDA medium to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7), and *T. harzianum* (Jn 14). Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of

mycelia. The spore concentration was then set to  $1 \times 10^6$  spores/ml with the aid of a haemocytometer [Tiefe Depth Protondeur 0.200 mm]. A total of 20  $\mu$ l of the fungal suspension were placed in the middle of each of the 24 Sarstedt microtitre plate wells (Sarstedt, Newton, USA), (Figure 4) in addition to 480  $\mu$ l of each sugar solution to reach a final volume of 500  $\mu$ l. The cultures were then incubated under continuous light for 48 hours at  $25 \pm 1^\circ \text{C}$ . Spore germination counts were done using an inverted microscope (Figure 5) (Doehlemann *et al*, 2006). Randomly selected conidia (100 conidia) were counted in each of the 4 wells sampled under inverted microscope. A spore was considered as germinated when the germ tube length was equal and/or exceeding the conidial diameter. At the same time, the average germ tube lengths of 10 random germinated conidia (replicates) were recorded ( $\mu\text{m}$ ) using a calibrated inverted microscope eyepiece graticule. A completely randomized design was used with 8 replicate for each treatment. Each experiment was repeated 3 times.



**Figure 4.** Sarstedt microtitre plate (Sarstedt, Newton. USA), used throughout the study for germination assays of *Fusarium* and *Trichoderma* conidia



**Figure 5.** Inverted microscope (Olympus® Hamburg, Germany) used throughout this study for monitoring germination of *Fusarium* and *Trichoderma* conidia.

### **2.2.2 Effect of inorganic nitrogen sources.**

The effect of inorganic nitrogen sources (Ammonium and Nitrate) on conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was investigated using  $\text{NH}_4$  and  $\text{NO}_3$  in six molar concentrations (0.001 mM, 0.01 mM, 0.1 mM, 1 mM, 10 mM, and 100 mM). Solutions were prepared in distilled water and sterilized in the autoclave for 30 minutes at 127°C. Both fungi were grown on PDA medium to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum*, and *T. harzianum*. Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of mycelia. The spore concentration was then set to  $1 \times 10^6$  spores/ml with the aid of a haemocytometer [Tiefe Depth Protondeur 0.200 mm]. A total of 20  $\mu\text{l}$  of the fungal suspension were placed in the middle of each of the 24 Sarstedt microtitre plate wells (Sarstedt, Newton, USA), in addition to 480  $\mu\text{l}$  of each nitrogen source solution to reach a final volume of 500  $\mu\text{l}$ . The cultures were then incubated under continuous light for 48 hours at  $25 \pm 1^\circ\text{C}$ . Spore germination counts were then done using an inverted microscope. Randomly selected conidia (100 conidia) were counted in each of the 4 wells

sampled under inverted microscope. At the same time, the average germ tube lengths of 10 random germinated conidia (replicates) were measured ( $\mu\text{m}$ ) using a calibrated inverted microscope eyepiece graticule. A completely randomized design was used with 8 replicate for each treatment. Each experiment was repeated 3 times.

### **2.2.3 Effect of Cations.**

The effect of the cations  $\text{K}^+$ ,  $\text{Ca}^{+2}$ , and  $\text{P}^+$  on conidial germination and germ tube length of *T. harzianum isolates* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was investigated. Ca ( $\text{CaCl}_2$ ), K (KCl) and P ( $\text{K}_2\text{HPO}_4$ ) were prepared into seven concentrations (0.001mM, 0.01mM, 0.1mM, 1 mM, 10 mM, 100 mM, and 1000 mM). Solutions were prepared in distilled water and sterilized in the autoclave for 30 minutes at  $127^\circ\text{C}$ . Both fungi were grown on PDA medium to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of both fungi. Conidia were then harvested from the surface of the cultures by scraping fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of mycelia. The spore concentration was then set to  $1 \times 10^6$  spores/ml with the aid of a haemocytometer [Tiefe Depth

Protondeur 0.200 mm]. A total of 20 $\mu$ l of the fungal suspension were placed in the middle of each of the 24 Sarstedt microtitre plate wells (Sarstedt, Newton, USA), in addition to 480 $\mu$ l of each cation solution to reach a final volume of 500 $\mu$ l. The cultures were then incubated under continuous light for 48 hours at  $25 \pm 1^\circ\text{C}$ . Spore germination counts were then done using an inverted microscope. Randomly selected conidia (100 conidia) were counted in each of the 4 wells sampled under inverted microscope. At the same time, the average germ tube lengths of 10 random germinated conidia (replicates) were recorded ( $\mu\text{m}$ ) using a calibrated inverted microscope eyepiece graticule. A completely randomized design was used with 8 replicate for each treatment. Each experiment was repeated 3 times.

**2.3 The effect of the exogenously applied nitrogen forms (NH<sub>4</sub>, NO<sub>3</sub>), and Cations (K<sup>+</sup>, Ca<sup>2+</sup>, P<sup>+</sup> and Fe<sup>2+</sup>) on biocontrol efficiency of *T. harzianum* (Jn 14) against *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7).**

**2.3.1 Soil Preparation**

Soil samples were collected from Hebron university greenhouse, and passed through a 4 mm mesh sieve to remove plant residues and gravels. Sieved samples were autoclaved for 30 minutes at 127°C.

**2.3.2 Fusarium wilt Disease severity**

The effect of *T. harzianum* (Jn 14) on Fusarium wilt of tomato plant in nutrient amended soils was assessed. Soil was amended with inorganic nitrogen forms (NH<sub>4</sub>, NO<sub>3</sub>), and the cations (K<sup>+</sup>, Ca<sup>2+</sup>, P<sup>+</sup> and Fe<sup>2+</sup>). Three concentrations from each nutrient were prepared (0, 1, 10 and 100µM). Solutions were prepared in distilled water and autoclaved for 30 minutes at 127°C. *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) were grown on PDA medium to which 1.5% Streptomycin sulphate added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum*, and *T. harzianum*. Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of mycelia. The spore suspension concentrations for both fungi were calibrated at 1\*10<sup>6</sup> conidia/ml for *F. oxysporum* f. sp.

*lycopersici* and  $1 \times 10^9$  conidia/ml for *T. harzianum* (Jn14) using a haemocytometer [Tiefe Depth Profondeur, 0.200 mm].

For soil inoculation, 1 ml of the spore suspension stock solution was used to inoculate 1 Kg of soil to have a final conidial concentration of  $10^3$  conidia/gm soil for *F. oxysporum* f. sp. *lycopersici* and  $10^6$  conidia/gm soil for *T. harzianum* (Jn14). The experimental treatments involved were: two control (CK) treatments with and without *T. harzianum* (Jn14) using 0 concentration of each nutrient, and then the use of each nutrient at the three concentrations (1, 10 and 100 $\mu$ M). All treatment involved of course the soil inoculation with  $10^3$  conidia/ gm of *F. oxysporum* f. sp. *lycopersic*.

Tomato plants (Cv.144R) in the age of 40 days were transplanted in pots containing 1000gm of treated soil each. Pots were incubated in greenhouse for 3 months after which disease severity was assessed (% of dead plants).

### **2.3.3 Effect of nitrogen forms (NH<sub>4</sub>, NO<sub>3</sub>) on population of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in soil.**

The effect of inorganic nitrogen sources (NH<sub>4</sub> and NO<sub>3</sub>) on population (CFU/g) of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in soil was investigated using three molar concentrations (0, 1, 10 and 100µM). Solutions were prepared in distilled water and autoclaved for 30 minutes at 127°C. *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) were then grown on PDA medium to which 1.5% Streptomycin sulphate added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum*, and *T. harzianum*. Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in to remove all traces of mycelia. The spore suspension concentrations for both fungi were calibrated at 1\*10<sup>6</sup> conidia/ml for *F. oxysporum* f. sp. *lycopersici* and 1\*10<sup>9</sup> conidia/ml for *T. harzianum* (Jn14) using a haemocytometer [Tiefe Depth Protondeur 0.200 mm].

For soil inoculation, 1 ml of the spore suspension stock solution was used to inoculate 1 Kg of soil to have a final conidial concentration of 10<sup>3</sup> conidia/gm soil for *F. oxysporum* f. sp. *lycopersici* and 10<sup>6</sup> conidia/gm soil for *T.*

*harzianum* (Jn14). The experimental treatments involved were: control (CK) treatment using 0 concentration of each nutrient, and then the use of each nutrient at the three concentrations (1, 10 and 100 $\mu$ M).

Tomato plants (Cv.144R) in the age of 40 days were transplanted in pots containing 1000gm of treated soil each. Pots were incubated in greenhouse for 3 months. The variously treated soils in the pots were later used to determine the Foxy and the *T. harzianum* (Jn14) populations (cfu g<sup>-1</sup>).

After three months, both fungi were recovered from the amended soil using the dilution plate technique on a *Trichoderma*-selective medium (TSM) for *T. harzianum* (Elad *et al.*, 1981), and on *Fusarium* selective peptone-PCNB agar medium for Foxy (Nelson *et al.*, 1983), according to the following procedure: a 50 g air-dried soil sample was suspended in 500 mL and shaken for 20–30 min. in a rotary shaker at 200 rpm. Serial dilutions (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup>) were made of each soil sample, and 200  $\mu$ l of a 10<sup>-3</sup> soil suspension was plated onto 90-mm diam. Petri dishes containing TSM and *Fusarium* selective peptone-PCNB. The plates were then incubated for 5–7 days at 25°C, and the colonies were counted. A completely randomized design was used with 8 replicate for each treatment. Each experiment was repeated 3 times.

#### **2.3.4 Effect of Cations ( $K^+$ , $Ca^{2+}$ , $P^+$ and $Fe^{2+}$ ) on *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) population (CFU/g) in soil.**

The effect of the cations ( $K^+$ ,  $Ca^{2+}$ ,  $P^+$  and  $Fe^{2+}$ ) on population (CFU/g) of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in soil was investigated using three molar concentrations (0, 1, 10 and 100 $\mu$ M). Solutions were prepared in distilled water and autoclaved for 30 minutes at 127°C. *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) were grown on PDA medium to which 1.5% Streptomycin sulphate added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum*, and *T. harzianum*. Conidia were then harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of mycelia. The spore suspension concentrations for both fungi were calibrated at  $1 \times 10^6$  conidia/ml for *F. oxysporum* f. sp. *lycopersici* and  $1 \times 10^9$  conidia/ml for *T. harzianum* (Jn14) using a haemocytometer [Tiefe Depth Profondeur 0.200 mm].

For soil inoculation, 1 ml of the spore suspension stock solution was used to inoculate 1 Kg of soil to have a final conidial concentration of  $10^3$  conidia/gm soil for *F. oxysporum* f. sp. *lycopersici* and  $10^6$  conidia/gm soil for *T.*

*harzianum* (Jn14). The experimental treatments involved were: control (CK) treatment using 0 concentration of each nutrient, and then the use of each nutrient at the three concentrations (1, 10 and 100 $\mu$ M).

Tomato plants (Cv.144R) in the age of 40 days were transplanted in pots containing 1000gm of treated soil each. Pots were incubated in greenhouse for 3 months. The variously treated soils in the pots were later used to determine the Foxy and the *T. harzianum* (Jn14) populations (cfu g<sup>-1</sup>).

After three months, both fungi were recovered from the amended soil using the dilution plate technique on a *Trichoderma*-selective medium (TSM) for *T. harzianum* (Elad *et al.*, 1981), and on *Fusarium* selective peptone-PCNB agar medium for Foxy (Nelson *et al.*, 1983), according to the following procedure: a 50 g air-dried soil sample was suspended in 500 mL and shaken for 20–30 min. in a rotary shaker at 200 rpm. Serial dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$ ) were made of each soil sample, and 200  $\mu$ l of a  $10^{-3}$  soil suspension was plated onto 90-mm diam. Petri dishes containing TSM and *Fusarium* selective peptone-PCNB. The plates were then incubated for 5–7 days at 25°C, and the colonies were counted. A completely randomized design was used with 8 replicate for each treatment. Each experiment was repeated 3 times.

### **2.3.5 The effect of nitrogen forms (NH<sub>4</sub> and NO<sub>3</sub>) in the presence of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) on tomato plant's growth parameters.**

The effect of inorganic nitrogen forms (NH<sub>4</sub> and NO<sub>3</sub>) in combination with *T. harzianum* (Jn14) in the presence of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) on tomato plant's growth (plant height, dry weight, and fresh weight) was investigated using three molar concentrations (0, 1, 10 and 100µM). Solutions were prepared in distilled water and autoclaved for 30 minutes at 127°C. *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) were grown on PDA medium to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum*, and *T. harzianum*. Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of mycelia. The spore suspension concentrations for both fungi were calibrated at 1\*10<sup>6</sup> conidia/ml for *F. oxysporum* f. sp. *lycopersici* and 1\*10<sup>9</sup> conidia/ml for *T. harzianum* (Jn14) using a haemocytometer [Tiefe Depth Protondeur 0.200 mm].

For soil inoculation, 1 ml of the spore suspension stock solution was used to inoculate 1 Kg of soil to have a final conidial concentration of 10<sup>3</sup> conidia/

gm soil for *F. oxysporum* f. sp. *lycopersici* and  $10^6$  conidia/gm soil for *T. harzianum* (Jn14). The experimental treatments involved were: control (CK) treatment using 0 concentration of each nutrient, and then the use of each nutrient at the three concentrations (1, 10 and 100 $\mu$ M).

Tomato plants (Cv.144R) in the age of 40 days were transplanted in pots containing 1000gm of treated soil each. Pots were incubated in greenhouse for 3 months after which plant growth parameters were measured. Plant heights were recorded in cm from soil surface to the apex. Concerning fresh and dry weights, plants were washed under running tap water to remove soil from roots; plants were then dried at 80°C in drying oven after recording fresh weights (gm). After 72 hr, plant dry weights (gm) were determined (Shenker *et al.*, 1992).

## **2.4 Induced Plants Growth Response**

The ability of *T. harzianum* (Jn14) to induce an increase in plant height, fresh weight, dry weight, and flowering of tomato plant was tested. *T. harzianum* (Jn14) was grown on PDA medium for 10 days to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations. Spore suspension was then prepared from 10 days old cultures of *T. harzianum* (Jn14). Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove any traces of mycelia. The spore suspension concentrations were calibrated at  $1 \times 10^9$  conidia/ml for *T. harzianum* (Jn14) using a haemocytometer [Tiefe Depth Protondeur 0.200 mm].

For soil inoculation, 1 ml of the spore suspension stock solution was used to inoculate 1 Kg of soil to have a final conidial concentration of  $10^6$  conidia/gm soil for *T. harzianum* (Jn14). The experimental treatments involved were: control (CK) treatment without *T. harzianum* (Jn14) and the treatment with *T. harzianum* (Jn14). Tomato plants (Cv.144R) in the age of 40 days were transplanted in pots containing 1000gm of treated soil each. Pots were incubated in greenhouse for 3 months after which plant growth parameters, including number of flowers were measured. Plant heights were recorded in cm from soil surface to the apex. Concerning fresh and dry

weights, plants were washed under running tap water to remove soil from roots; plants were then dried at 80°C in drying oven after recording fresh weights (gm). After 72 hr, plant dry weights (gm) were determined (*Shenker et al., 1992*).

## **2.5 Enzyme's production by *T. harzianum***

### **2.5.1 Chitinolytic activity**

#### **2.5.1.1 Preparation of colloidal chitin**

Rodriguez-Kabana *et al*, 1983 described the method for the preparation of colloidal chitin. Colloidal chitin suspension was prepared by dissolving 200g of ground (0.25mm) crustacean chitin (sigma C3387) in 1.8 liter of concentrated HCl. The acid was added to the chitin with stirring, and the mixture was allowed to stand at 20°C with intermittent stirring until completely dissolved (1.5-2h). The solution was poured into a 60-liter container half filled with tap water. A suspension of chitin in water was formed and more water was added to have a final volume of 50 liters. The suspension was allowed to stand overnight to allow chitin to settle and form a concentrated suspension. The supernatant liquid was slowly siphoned out and tap water was repeatedly added for 4 times in the same manner, followed by 3 washes with distilled water. After the final wash, the suspension was passed through a triple layer of 0.1 mm mesh nylon cloth to remove large particles. The resulting chitin suspension had a pH of 5.5-6.0 and was stored in the dark at 4°C until used. Chitin content of the suspension was determined gravimetrically after drying 10 ml sample at 80°C for 42hr. The stock suspension was used to prepare other lower concentrations by diluting with distilled water immediately prior to use.

### **2.5.1.2 Preparation and extraction of proteins**

The objective of this procedure was to separate the *T. harzianum* (Jn 14) and *T. asperillum* (T203) mycelium biomass from the supernatant which will be used for the extracellular protein extraction. The mycelium biomass of *T. harzianum* (Jn 14) and *T. asperillum* (T203) was also used for the intracellular protein extraction. *T. harzianum* (Jn 14) and *T. asperillum* (T203) were grown for two days in PDB. The mycelia were then transferred aseptically into SM medium (Haran *et al*, 1995) with 0.5% C (colloidal chitin) for lytic enzymes induction and incubated for two days at 25°C. The culture filtrate were concentrated using a rotavapor (Germany); the filtrate was then used as extracellular protein extract for chitinolytic activity assays. Intracellular protein extracts were obtained from frozen mycelia growing in SM medium with colloidal chitin. The mycelia were crushed with silica beads (0.5mm) in activity buffer (100 mM potassium acetate buffer pH4.5) and protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Germany). The mixture was then centrifuged at high speed (5000 rpm) for 10 minutes and the supernatant was then taken and used as the protein extract.

### **Total protein concentration assay**

The objective of this procedure was the purification of protein to determine the amount of enzymatic activity vs. protein concentration. The total protein concentration was determined according to the method by Bradford, 1976 at

an absorbance length of 595 nm against a Bovine Serum Albumin (BSA) standard curve. Protein standard was prepared using Bovine Serum Albumin (BSA) with concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1mg/ml for standard assay.

**Microassay procedure** – To determine the concentration of the protein samples, a graph must be prepared. A 0.2 ml of dye stock solution and 0.8 ml of one of the protein standard solutions or samples were added in a 1.4 ml plastic cuvette to be assayed. This was mixed and the absorbance was noted at 595 nm. A graph was prepared with (protein concentration) vs. (absorbance at 595 nm) for the protein standard.

### **2.5.1.3 Enzymatic activity assay**

Chitinases can be classified into two major categories: Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases, however, can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin microfibril, and  $\beta$ -(1,4) N-acetyl glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manocha 1993).

## **Exochitinolytic assay**

The exochitinolytic assay was done according to the method of **Tronsmo and Harman, (1993)**. The substrates used for the assay are 4 Nitrophenyl N-acetyl- $\beta$ -D-glucosaminide (dimer) and 4 Nitrophenyl N, N-diacetyl- $\beta$ -D-chitobioside (trimer) (Sigma N9376 and N6133, respectively). Dimer and trimer substrates were prepared at the concentration of 0.3 mg/ml in potassium phosphate buffer 50 mM pH 4.5. Test assays were prepared by adding 90 $\mu$ l of each substrate with 30 $\mu$ l of the protein extract from *T. harzianum* (Jn 14) and *T. asperillum* (T203) (sample adjusted according to the protein concentration with phosphate buffer). Protein concentration of samples was 1 mg/ml. The samples were concentrated using Centricon (Germany). The standard solution was prepared by adding 1 $\mu$ l of mM of p-Nitrophenol standard solution (Sigma N7660) with 999 $\mu$ l of stop solution. This was vortexed briefly and stored in ice to inhibit enzyme activity. Each assay needed 120 $\mu$ l of the standard solution. The blank was prepared by using 30 $\mu$ l of each substrate with 60 $\mu$ l of potassium phosphate buffer. The plates were incubated for 30 minutes at 37°C. Finally, 30 $\mu$ l of stop solution (Sodium Carbonate, 0.2g/ml) was added to have a final volume of 120 $\mu$ l. Stop solution was added so the color becomes slightly yellow if chitinase

enzyme present. The absorbance was measured at 595nm (not later than 30 minutes after ending the reaction) using a fluorescent spectrophotometer.

Chitinase Activity calculation;

$$\text{Units/ml} = (A_{595} \text{ sample} - A_{595} \text{ blank}) \times 0.01 \times 0.12 / A_{595} \text{ standard} \times V_{\text{enz}}$$

Where  $A_{595}$  sample: absorbance of the sample at 595nm.

$A_{595}$  blank -absorbance of the blank at 595nm.

0.01:  $\mu\text{mole/ml}$  of p-Nitrophenol in the standard solution

0.12: final volume in milli liters of the reaction after the addition of stop solution.

$A_{595}$  standard: absorbance of the standard at 595nm

Time: minutes

$V_{\text{enz}}$ : volume of the sample in milli liter

## **Endochitinolytic assay**

Endochitinase activity was detected by monitoring the release of reducing groups from colloidal chitin, which was measured by the reduction of turbidity of the colloidal chitin suspension (Tronsmo and Harman, 1993).

Colloidal chitin (1% w/v of moist purified chitin) was suspended in 50 mM potassium phosphate (pH 6.7). This suspension (0.5ml per assay) was added to an equal volume of test solution (0.5ml/sample) with the same protein concentration. The total protein concentration was determined by the method of Bradford (1976).

The positive control was 0.5 ml of colloidal chitin suspension with equal volume of potassium phosphate buffer and the negative control was 1 ml of potassium phosphate buffer alone. The two controls were incubated at 25°C for 24 hours and subjected to the reducing group assay described by Ashwell, (1957) as modified by Hayes, (1989). The mixture was diluted with 5 ml of water, and turbidity was determined at 510 nm. The percentage reduction in turbidity was plotted against the samples.

#### 2.5.1.3.1 Enzymatic assay on microtiter plate using fluorescent substrate

The assay was performed on cultures growing in microtiter plates, with a fluorogenic substrate: 4-methylumbelliferyl-N-acetyl-D-glucosaminide (4-MeUNAG). The fluorescence of the product, 4-methylumbelliferone, was detected. This method was successfully used to follow induction and repression of extracellular exochitinase activity in the biocontrol fungus *T. harzianum*. The method used was described by Carmi *et al*, (2001) and earlier by Haran *et al*, (1995). Chitinolytic enzymes can be detected *in-situ* using a set of three highly sensitive substrates (Sigma, MO, USA) that produce a fluorescent product following enzymatic hydrolysis:

- a) 4-methylumbelliferyl-N-acetyl- $\beta$ -D-glucosaminide [4-MU-(GlcNAc)]
- b) 4-methylumbelliferyl- $\beta$ -D-N-N'-diacetylchitobioside [4-MU (GlcNAc)<sub>2</sub>]
- c) 4-methylumbelliferyl- $\beta$ -D-N-N'-N''-triacetylchitotriose [4-MU-(GlcNAc)<sub>3</sub>]

These compounds function as dimeric, trimeric, and tetrameric substrates respectively with the 4-methylumbelliferyl group linked by  $\beta$ -1,4 linkage to the N-acetylglucosamine oligosaccharides. Hydrolysis of this linkage yields the fluorescent product 4-methylumbelliferone visualised under UV.

#### **2.5.1.3.2 Colloidal chitin as inducer**

*T. harzianum* (Jn14) and *T. asperillum* (T203) were grown in PDB and after two days transferred aseptically into SM medium with colloidal chitin (C). Cultures were incubated at 25°C for 2 days. The culture filtrates were concentrated (almost 100 fold) using Vivaspin (**Vivascience, Germany**) with a cut-off of 10,000 MWCO. These were tested for chitinolytic activity using dimeric and trimeric substrates (1µg/µl) (**Barboza Corona et al, 1999**). Later, a 21µl (3µg) of *T. harzianum* (Jn14) and 16µl (3µg) of *T. asperillum* (T203) coming from intracellular protein, in addition to 19.2µl (5µg) of *T. asperillum* (T203), and 31µl (5µg) of *T. harzianum* (Jn14) coming from extracellular protein were added in microtiter plates. The total protein concentration was determined by the method of **Bradford (1976)**.

The plates were visualized under UV immediately, after 30 minutes and after 60 minutes to observe the production of fluorescence. The negative control was supernatant of the two fungi with glucose, and the crude culture filtrate from *T. harzianum* (Jn14) and *T. asperillum* (T203).

## 2.5.2 $\beta$ -1,3 Glucanase activity

### 2.5.2.1 Enzymatic activity assay

The goal of this experiment was glucanase induction from *T. harzianum*.

The  $\beta$ -1, 3 glucanase assays was determined by the method of Nelson, (1957) and Somogyi, (1952), monitoring the amount of reducing sugars released from laminarin (Sigma L9634). Laminarin solution was prepared (1mg/ml) in potassium phosphate buffer 50 mM at pH 6.7. In an eppendorf or polypropylene falcon tube. 500 $\mu$ l of the above laminarin solution and 500 $\mu$ l of protein extract (sample adjusted according to the protein concentration with phosphate buffer), were mixed and kept for agitation at 30°C for 2 hr. The samples used were extra cellular protein extracts from the different strains grown in SM medium.

The standard glucose solutions (4mg/ml) were prepared with different concentrations of 2, 4, 6, 8, 10  $\mu$ g/ml. Later, copper reagent was added to all the standard solutions and samples. This was vortexed and incubated at 120°C for 20 minutes (crucial step is at 120°C for liberation of reducing sugars). The samples were incubated in ice for 10-15 minutes. Later, 500 $\mu$ l of arsenomolybdate solution was added, vortexed and made up the volume to 8.5 ml with water. The absorbance measured at normal (tube) spectrophotometer.

$\beta$ -1,3 glucanase activity calculation:

$$\text{Activity (mmol/1min.)} = 1000 \times C_g (V_r \times t \times V_e \times M_g)$$

$$\text{Specific activity (mmol/mg protein .min)} = 1000 \times C_g (V_r \times t \times C_p \times V_e \times M_g)$$

Where  $C_g$  -concentration of glucose (mg/ml)

$C_p$  -protein concentration ( $\mu$ g/ml)

$V_r$  -volume of reaction (1000  $\mu$ l)

$t$  -time point (min)

$V_e$  -volume of the enzyme (500 $\mu$ l)

$M_g$  -molecular weight of glucose (180gm/ml)

**2.5.2.1.1 Laminarin as inducer**

*T. harzianum* (Jn14), and *T. harzianum* (Jn58), were grown in PDB and after two days transferred aseptically into SM medium with 1 mg/ml laminarin.

The culture filtrates (extra cellular protein extracts) were concentrated almost 100 fold using Vivaspin (Vivascience ,Germany) with a cut off of 10,000 MWCO. Later, 100  $\mu$ l protein extract, and 400  $\mu$ l of laminarin were added together, and incubated at 37°C for 1hr, boiled for 5 min, and finally the glucose on normal spectrophotometer was measured.

### **2.5.3 Effect of *T. harzianum* (Jn 14) enzymes (chitinases and glucanases) supernatant on *F. oxysporum* f.sp. *lycopersici* (Lo7) conidial germination and germ tube growth in vitro.**

A number of cell wall degrading enzymes (CWDEs) and cell membrane affecting compounds (MACs) that alter cell membrane structure or permeability have been assayed in vitro against *F. oxysporum* f.sp. *lycopersici* (Lo7). The bioassay was performed essentially as described in [Lorito, et al, \(1996\)](#). *F. oxysporum* f.sp. *lycopersici* (Lo7) was grown on PDA medium to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations for 10 days. Spore suspensions were prepared from 10 days old cultures of *F. oxysporum* f.sp. *lycopersici* (Lo7) Conidia were harvested from the surface of the cultures by scraping the fungal colonies by a glass rod with 10 ml of sterile distilled water. The suspension was then filtered through four layers of sterile cheesecloth in order to remove all traces of mycelia. The spore concentration was then set to  $1 \times 10^6$  spores/ml with the aid of a haemocytometer [Tiefe Depth Protondeur 0.200 mm]. A total of 30µl containing 2000-3000 conidia of *F. oxysporum* f.sp. *lycopersici* (Lo7) were added in a flat-bottomed ELISA plate containing PDB media. The supernatant extract from *T. harzianum* (Jn14) (chitinases and glucanases) was added to the reaction mixture, after dialysis against buffer potassium acetate 0.1 M pH 5.5. The cultures were then incubated under

continuous light for 48 hours at  $25 \pm 1^\circ\text{C}$ . Spore germination counts were then done using an inverted microscope. Randomly selected conidia (100 conidia) were counted in each of the 4 wells sampled under inverted microscope. At the same time, the average germ tube lengths of 10 random germinated conidia (replicates) were recorded ( $\mu\text{m}$ ) using a calibrated inverted microscope eyepiece graticule. A completely randomized design was used with 8 replicate for each treatment. Each experiment was repeated 3 times.

## **2.6 Statistical analysis**

Data of all experiments were analyzed statistically using one way analysis of variance (ANOVA); fisher least significant difference (LSD) test was used for mean separation (SigmaStat<sup>®</sup> 2.0 program, Germany).

### **3. RESULTS**

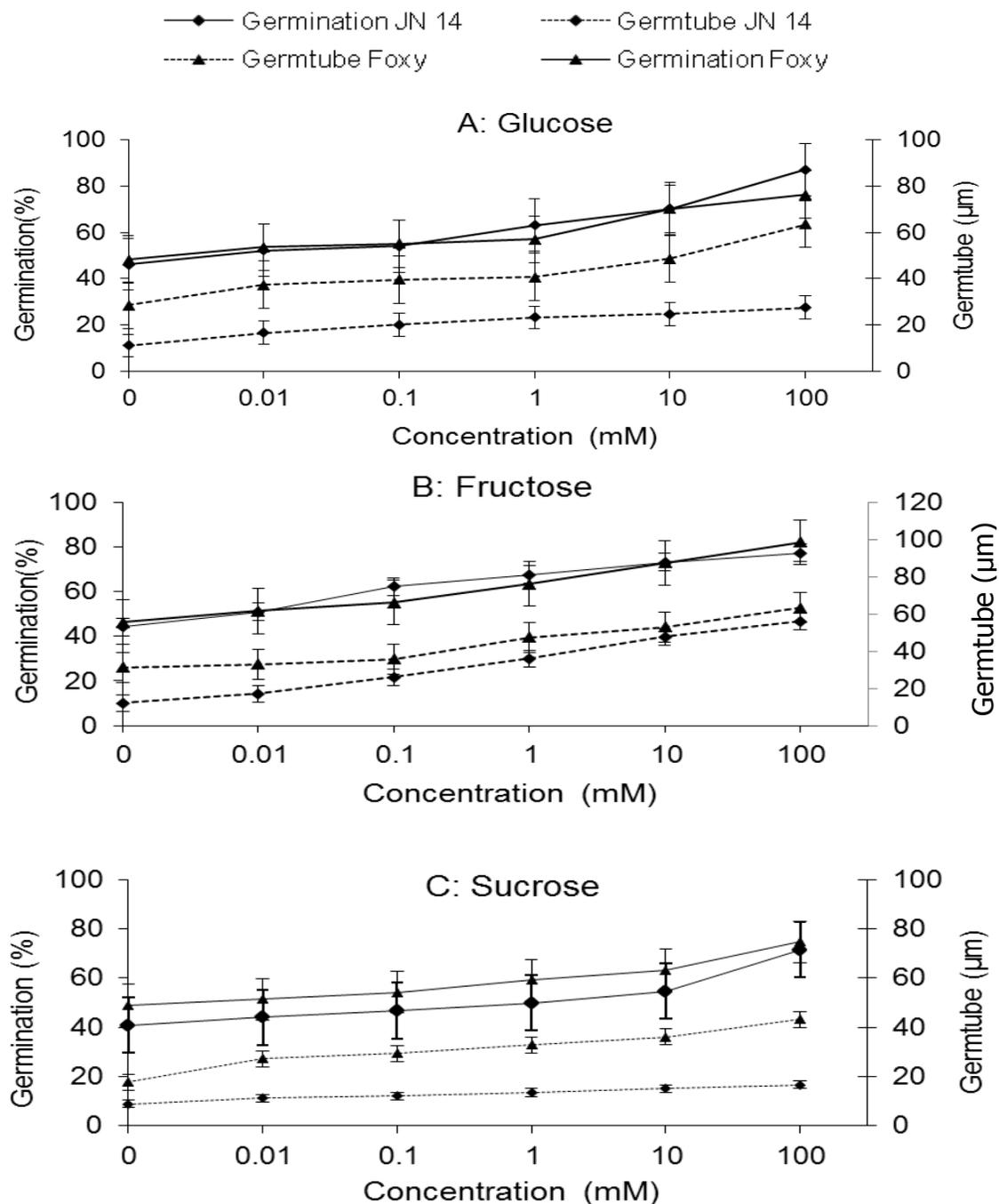
#### **3.1 The effect of the exogenously applied nutrients on conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in vitro.**

##### **3.1.1 The effect of Sugars (glucose, fructose and sucrose) on conidial germination of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in vitro.**

The effect of the sugars (Glucose, Fructose and Sucrose) on conidial germination and germ tube growth of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was tested at various concentrations (Figure 6).

In general, the results showed that there were no significant variations between Jn14 and Foxy in regards to their response to the three sugars tested. In addition, the results showed that germination of *T. harzianum* (Jn 14) conidia and germ tube growth were significantly stimulated in all sugars in various proportions especially at high concentrations compared to the control. Fructose stimulated the highest germination rate (93%) after 48 hours of incubation at the highest concentration used (100 mM) compared with sucrose (80%), and glucose (87%). This increase was significant compared to the control treatment which recorded (48%) germination. Concerning Foxy, the results showed that germination of Foxy conidia were also stimulated in all sugars but to lesser extents. Sucrose stimulated the highest germination rate (91%) after 48 hours of incubation at the highest concentration used (100 mM) compared with Fructose (82%), and glucose

(76%). As for germ tube elongation for **Foxy**, glucose has stimulated the highest germ tube growth rate (64 $\mu$ m) after 48 hours of incubation at the highest concentration used (100mM) compared with fructose (53 $\mu$ m), and sucrose (52 $\mu$ m). However, the germ tube elongation for **Jn14** showed slower development; fructose has stimulated the highest germ tube growth rate (47 $\mu$ m) after 48 hours of incubation at the highest concentration used (100mM) compared with sucrose (22 $\mu$ m), and glucose (27 $\mu$ m).

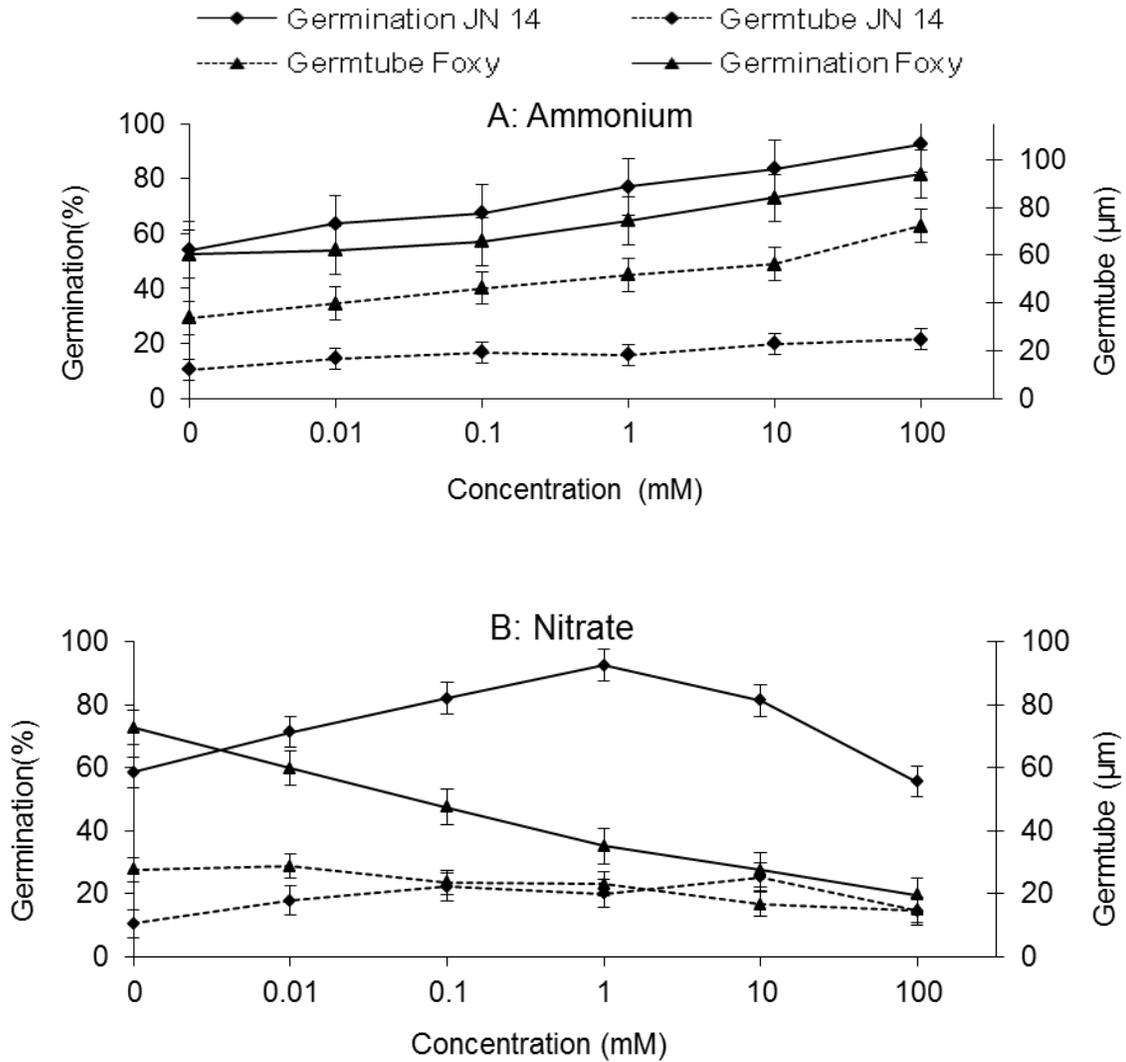


**Figure 6.** The effect of the exogenously applied sugars on conidial germination and germ tube elongation of *T. harzianum* (Jn 14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in vitro.

Treatment	LSD		
	Glucose	Fructose	Sucrose
Germination of Jn14:	11.169	4.737	11.226
Germination of Foxy:	10.208	10.111	8.517
Germtube elongation of Jn 14:	4.960	3.740	1.589
Germtube elongation of Foxy:	5.709	6.744	3.308

### **3.1.2 The Effect of inorganic nitrogen sources (NH<sub>4</sub>, NO<sub>3</sub>) on conidial germination (%) and germ tube length (µm) of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in vitro.**

The effect of NH<sub>4</sub> and NO<sub>3</sub> on conidial germination (%) and germ tube length (µm) of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was investigated (Figure 7). Results indicated that NH<sub>4</sub>-N enhanced conidia germination of *T. harzianum* (Jn14) significantly where it peaked at the highest concentration of 100mM (93%) compared with the control (54%) with no significant differences noticed between *T. harzianum* (Jn14) and Foxy. The germ tube growth, however, was not affected by the addition of NH<sub>4</sub>-N in *T. harzianum* (Jn14) but increased relatively in Foxy but to lesser extents than germination rates. Concerning NO<sub>3</sub>-N, results showed that this nitrogen form increased significantly germination rates of *T. harzianum* (Jn14) conidia (93%) at lower concentrations ( $\leq$  1mM). Germination rates started to decrease proportionally at NO<sub>3</sub> concentrations  $>$  1mM reaching its lowest (56%) at the highest concentration (100 mM). On the other hand, the addition of NO<sub>3</sub>-N has negatively influenced the germination of Foxy conidia at all concentrations compared with the control (73%). The germ tube growth of both fungi was not influenced, however, by the addition of this form of nitrogen.



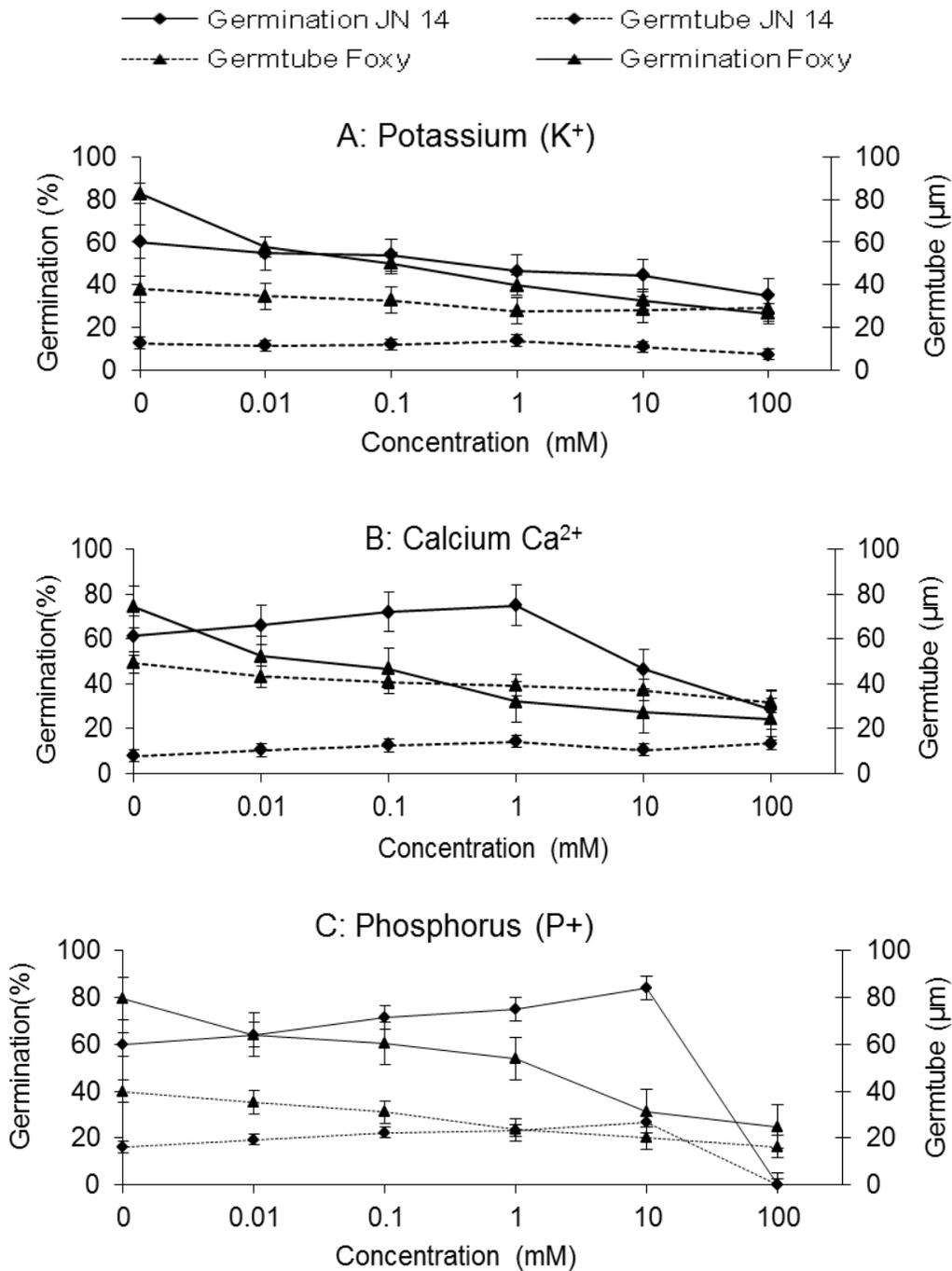
**Figure 7.** The effect of nitrogen forms on conidial germination (%) and germ tube length ( $\mu\text{m}$ ) of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy) after 48h of incubation at 25°C.

Treatment	LSD	
	Ammonium	Nitrate
Germination of Jn14:	10.322	4.919
Germination of Foxy:	8.687	5.517
Germtube elongation of Jn 14:	3.830	4.475
Germtube elongation of Foxy:	6.884	3.811

### **3.1.3 The Effect of the cations ( $K^+$ , $Ca^{2+}$ , and $P^+$ ) on conidial germination (%), and germ tube length ( $\mu m$ ) of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy) in vitro.**

The effect of the cations  $K^+$ ,  $Ca^{2+}$ , and  $P^+$  on conidial germination and germ tube elongation of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was investigated (Figure 8). Results indicated that  $K^+$  decreased germination rates of conidia of both fungi proportionally with increasing concentrations compared to the control with no significant differences between *T. harzianum* (Jn14) and Foxy. The germ tube growth, however, was not affected by the addition of  $K^+$  in both fungi. Concerning  $Ca^{2+}$ , results showed that  $Ca^{2+}$  increased germination rates of *T. harzianum* (Jn14) conidia (75%) at the concentrations ( $\leq 1mM$ ). Germination rates started to decrease proportionally at  $Ca^{2+}$  concentrations  $> 1mM$  reaching its lowest (24%) at the highest concentration (100 mM) compared with control (61%). On the other hand, the addition of  $Ca^{2+}$  has negatively influenced the germination of Foxy conidia at all concentrations compared with the control. The germ tube growth of both fungi was not influenced, however, by the addition of  $Ca^{2+}$ . Concerning  $P^+$ , results showed that  $P^+$  increased significantly germination rates of *T. harzianum* (Jn14) conidia (84%) at the concentrations ( $\leq 10mM$ ). Germination rates started to decrease proportionally at  $P^+$  concentrations  $> 10mM$  reaching its lowest (0%) at the

highest concentration (100 mM) compared with control (60%). On the other hand, the addition of  $P^+$  has negatively influenced the germination of Foxy conidia at all concentrations compared with the control. The germ tube growth of both fungi was not influenced by the addition of  $P^+$ .



**Figure 8.** The effect of cations on conidial germination (%) and germ tube length (µm) of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7).

Treatment	LSD		
	K <sup>+</sup>	Ca <sup>2+</sup>	P <sup>+</sup>
Germination of Jn14:	7.661	8.815	5.074
Germination of Foxy:	4.993	9.202	9.145
Germtube elongation of Jn 14:	2.599	2.808	2.345
Germtube elongation of Foxy:	6.187	4.934	4.858

### **3.2 The effect of the exogenously applied cations ( $\text{NH}_4$ , $\text{NO}_3$ , $\text{K}^+$ , $\text{Ca}^{2+}$ , $\text{Fe}^{2+}$ and $\text{P}^+$ ) on biocontrol efficiency of *T. harzianum* (Jn 14) against *F. oxysporum* f.sp. *lycopersici* in vivo.**

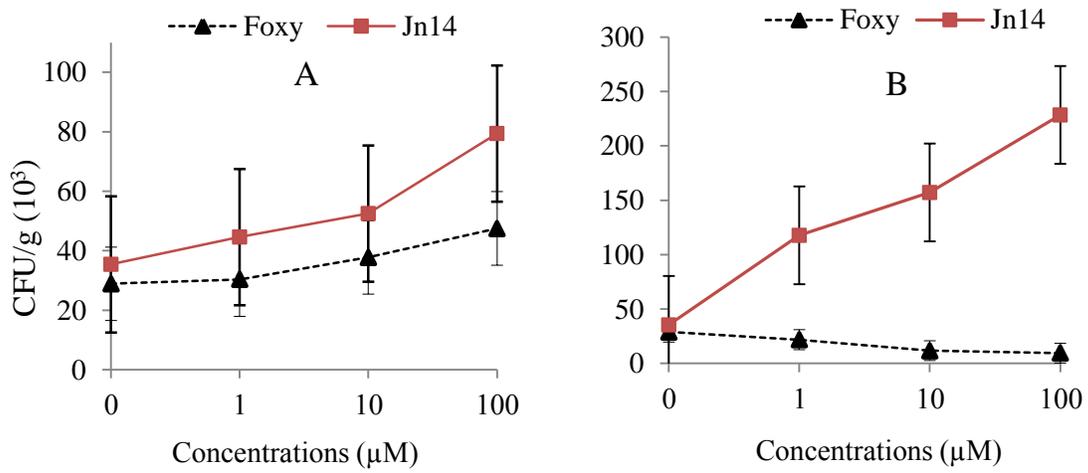
#### **3.2.1 *Fusarium* Disease severity**

The effect of *T. harzianum* (Jn 14) on Fusarium wilt of tomato plant in nutrient amended soils was investigated. The results showed that the nitrogen form ( $\text{NH}_4$  and  $\text{NO}_3$ ) and the cations ( $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{P}^+$ ) have not influenced the biocontrol efficiency of *T. harzianum* (Jn 14). All treatments that had the bioagent *T. harzianum* (Jn 14) recorded no disease at all (zero disease severity). However, the plants grown in Foxy amended soils with no *T. harzianum* (Jn 14) and no nutrient added recorded 36.5% disease severity.

### **3.2.2 Effect of nitrogen forms (NH<sub>4</sub> and NO<sub>3</sub>) on population of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in soil.**

The effect of inorganic nitrogen forms (NH<sub>4</sub> and NO<sub>3</sub>) on the population of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Lo7) was investigated (Figure 9). Results indicated that NH<sub>4</sub> increased the fungal population of both fungi in soil proportionally with increasing concentrations, but with no significant differences in general between *T. harzianum* (Jn14) and Foxy. However, at the highest concentration (100 mM) the population of *T. harzianum* (Jn14) recorded dramatic increase over the population of *F. oxysporum* f.sp. *lycopersici* (Lo7), but with no significant differences noticed with the control at all treatments.

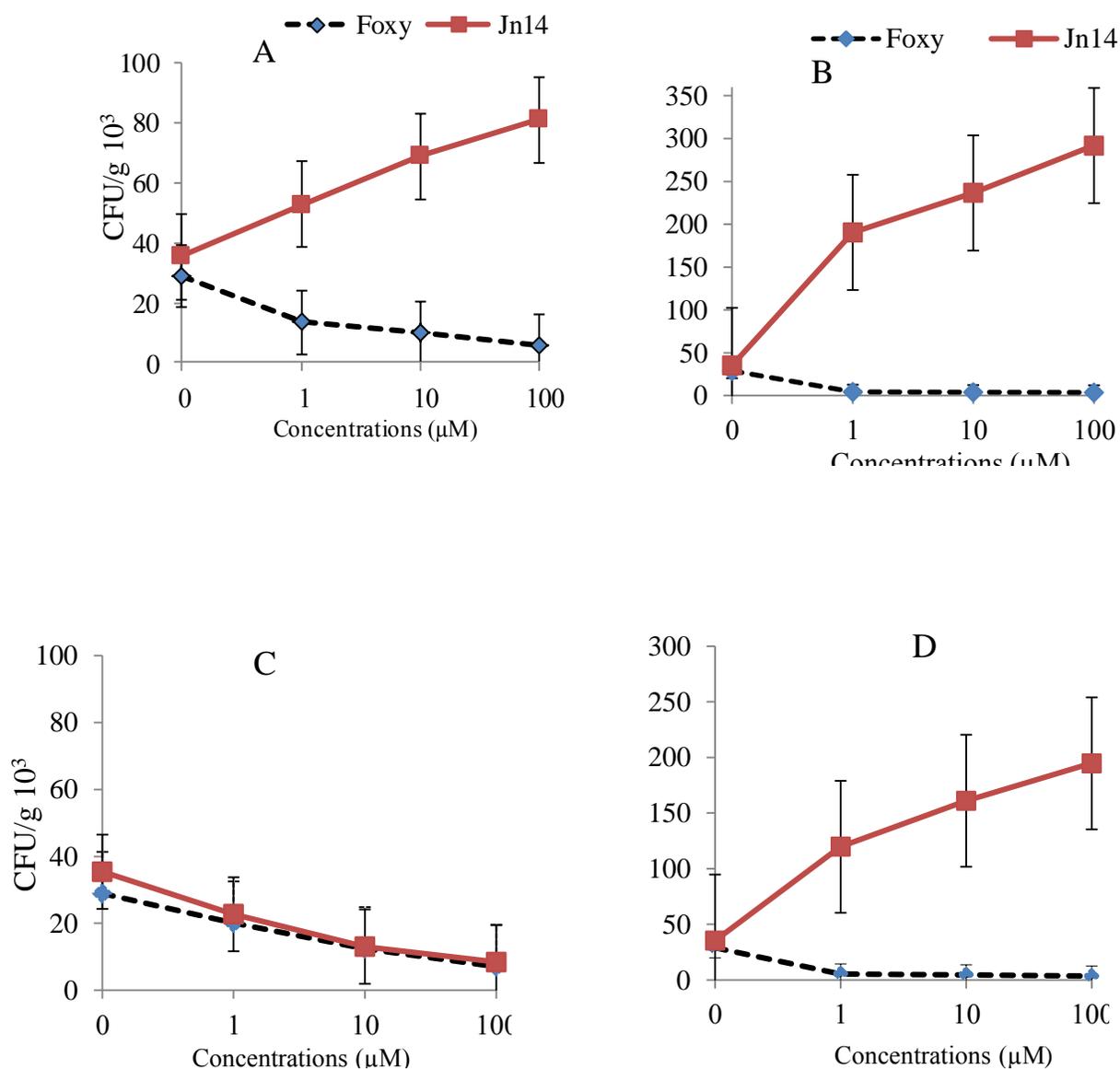
Concerning NO<sub>3</sub>, results showed that this nitrogen form increased significantly the population of *T. harzianum* (Jn14) at all concentrations. On the other hand, the addition of NO<sub>3</sub>-N has dramatically decreased the population of Foxy by (32%) at the highest concentration (100µM) compared with the control.



**Figure 9.** The effect of nitrogen forms on *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) population (CFU/g) in soil. **A:** Ammonium, (Jn14,  $\text{LSD}=22 \cdot 10^3$ ; Foxy,  $\text{LSD}=12 \cdot 10^3$ ) and **B:** Nitrate, (Jn14,  $\text{LSD}= 44 \cdot 10^3$ ; Foxy,  $\text{LSD}= 9 \cdot 10^3$ ).

### 3.2.3 The effect of cations ( $K^+$ , $Ca^{2+}$ , $P^+$ , $Fe^{2+}$ ) on *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) population in soil.

The effect of cations ( $K^+$ ,  $Ca^{2+}$ ,  $P^+$ ,  $Fe^{2+}$ ) on *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) population in soil was investigated (Figure 10). Results indicated that  $Ca^{2+}$ ,  $P^+$  and  $Fe^{2+}$ , increased the fungal population of *T. harzianum* (Jn14) in the soil significantly with increasing concentrations used, compared to Foxy and the control treatment. At the highest concentration (100  $\mu$ M) of these cations, the population of *T. harzianum* (Jn14) increased by (231%, 834%, 557%) and the population of *F. oxysporum* f. sp. *lycopersici* (Lo7) decreased by (17%, 13%, 12%), respectively compared with the control. However,  $K^+$  decreased the population of *T. harzianum* (Jn14) and Foxy with increasing concentrations with no significant differences between the two fungi.

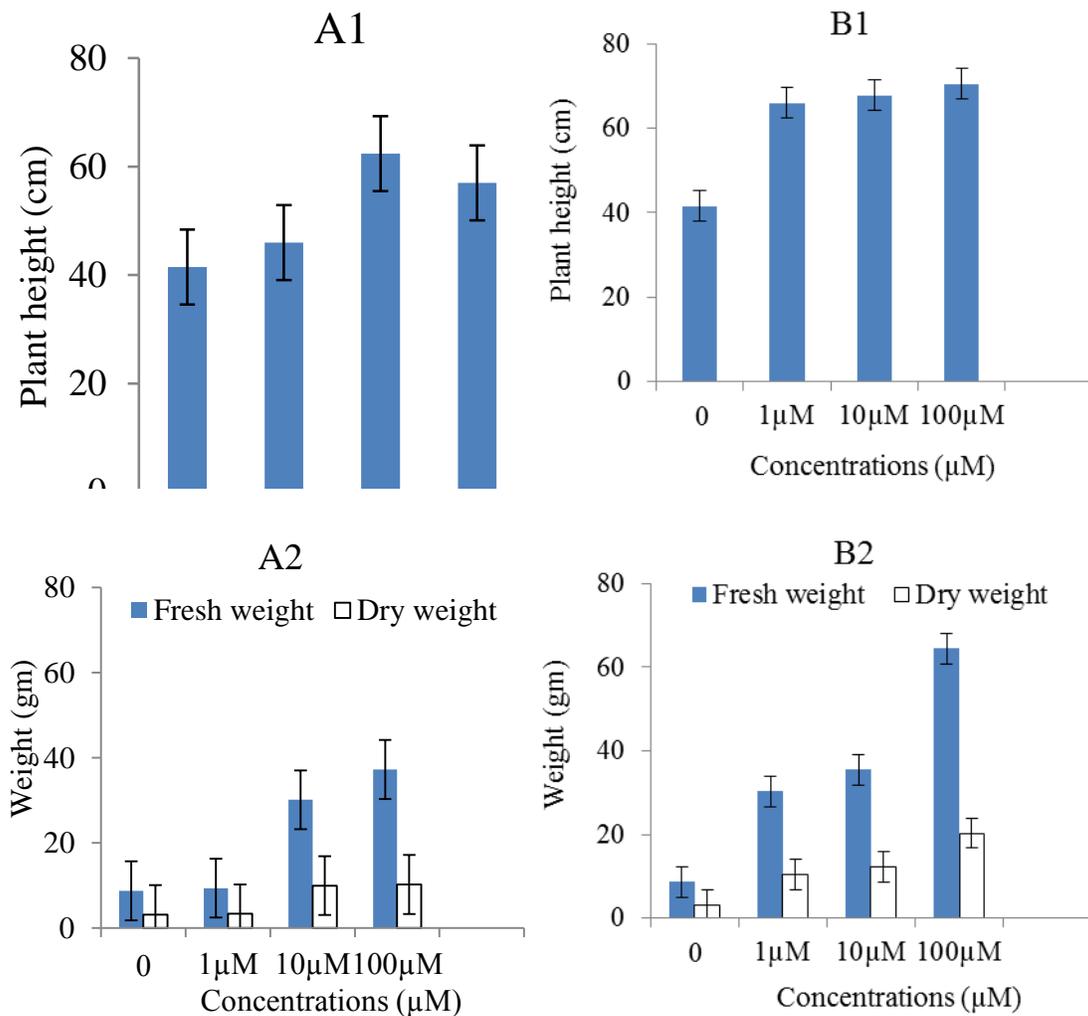


**Figure 10.** The effect of cations on *T. harzianum* (Jn14) and *F. oxysporum* f.sp. lycopersici (Foxy Lo7) population in soil.

[A: Calcium, Jn14:  $\text{LSD}=14 \times 10^3$ , Foxy:  $\text{LSD}=10 \times 10^3$ ]; [B: Phosphorus, Jn14:  $\text{LSD}=67 \times 10^3$ , Foxy:  $\text{LSD}=8 \times 10^3$ ]; [C: Potassium, Jn14:  $\text{LSD}=11 \times 10^3$ , Foxy:  $\text{LSD}=12 \times 10^3$ ] and [D: Ferrous, Jn14:  $\text{LSD}=59 \times 10^3$ , Foxy:  $\text{LSD}=8 \times 10^3$ ].

### **3.2.4 The effect of nitrogen forms (NH<sub>4</sub> and NO<sub>3</sub>) in the presence of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) on tomato plant growth parameters.**

The effect of inorganic nitrogen forms (NH<sub>4</sub> and NO<sub>3</sub>) in the presence of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) on tomato plant growth parameters was investigated (Figure 11). Results indicated that the addition of NH<sub>4</sub> and NO<sub>3</sub> has significantly improved tomato plant growth parameters in the presence of the two fungi. This was reflected positively on plant heights, fresh weights and dry weights. In addition, growth parameters increased with the increase of nitrogen forms concentrations. NH<sub>4</sub> and NO<sub>3</sub> at highest concentration (100 μM) have increased tomato plant heights by (135% and 169%), plant fresh weights by (411% and 711%), and dry weights by (333% and 666%), respectively compared with the control.



**Figure 11.** The effect of nitrogen forms ( $\text{NH}_4$  and  $\text{NO}_3$ ) in the presence of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) on tomato plant growth parameters.

[A1:  $\text{NH}_4$  (plant height), **LSD= 6.9**], [A2:  $\text{NH}_4$  (fresh weight), **LSD= 6.9**, A2:  $\text{NH}_4$  (dry weight), **LSD=2.6**]; [B1:  $\text{NO}_3$  (plant height), **LSD=3.6**], [B2:  $\text{NO}_3$  (fresh weight), **LSD= 8.1**, B2:  $\text{NO}_3$  (dry weight), **LSD= 3.5**].

### 3.3 Increased growth response of tomato plants induced by *T. harzianum* (Jn14).

The ability of *T. harzianum* (Jn14) to induce increased growth response in tomato plants (plant height, number of flowers, fresh and dry weights, and root fresh and dry weights) was investigated (Table 1). Results showed that there was a general increase in the values of growth parameters of tomato plants grown in soil amended with *T. harzianum* (Jn14) 3 months after sowing compared to the non-treated control. Tomato plant heights increased by (49%) compared with the control. However, flowering of plants was improved dramatically (> 5 fold) compared to the control. Concerning fresh and dry weights of plants, it was obvious that these parameters increased but with no significant differences statistically with the control.

**Table 1.** Increased growth response of tomato plants induced by *T. harzianum* (Jn14).

Treatment	Height (cm)	N. of flowers/plant	Fresh weight (gm)	Dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)
CK	28.625 b	0.250 b	10.175 a	3.513 a	1.587 b	0.537 a
Ck+Jn14	42.625 a	1.375 a	12.188 a	4.500 a	2.875 a	0.800 a
LSD	10.446	0.646	2.842	1.524	0.476	0.313

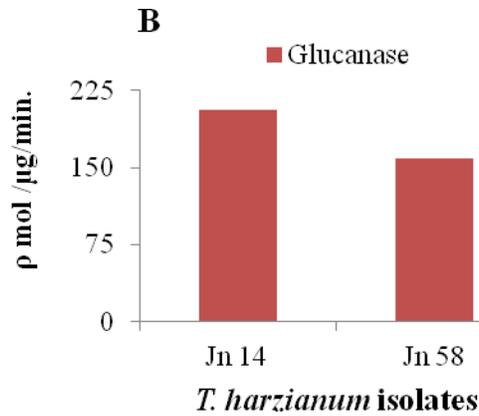
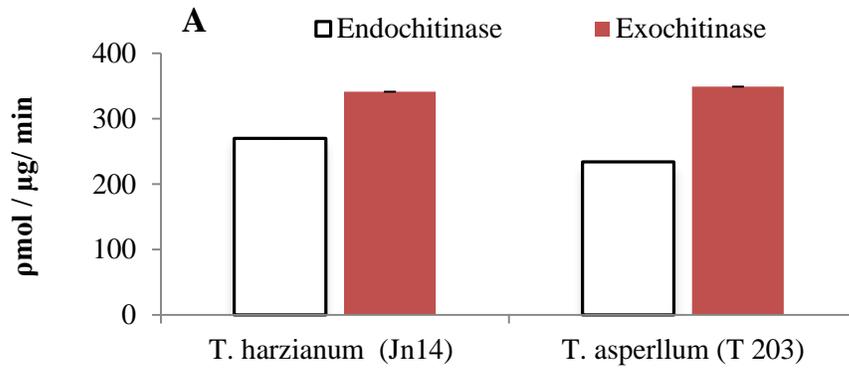
CK= tomato plant grown in soil without *T. harzianum* Jn14.

Ck+Jn14= tomato plant grown in soil amended with *Trichoderma harzianum* Jn14.

### **3.4 Enzyme's production by *T. harzianum* Jn14**

#### **3.4.1 Assessment of enzymes production (Chitinases and Glucanases) by *T. harzianum* (Jn 14).**

Assessment of enzymes production (Chitinases and Glucanases) by *T. harzianum* (Jn 14), and *T. asperillum* (T 203) was investigated (Figure 12). Results indicated that *T. harzianum* (Jn14) produced 15% more endochitinases than *T. asperillum* (T 203), while the later produced 2% more of the exochitinases, with no significant differences between the two fungi in the production of both chitinases. Concerning glucanase production by *T. harzianum*, results showed that the isolate Jn14 produced 21% more glucanase than the isolate Jn58.

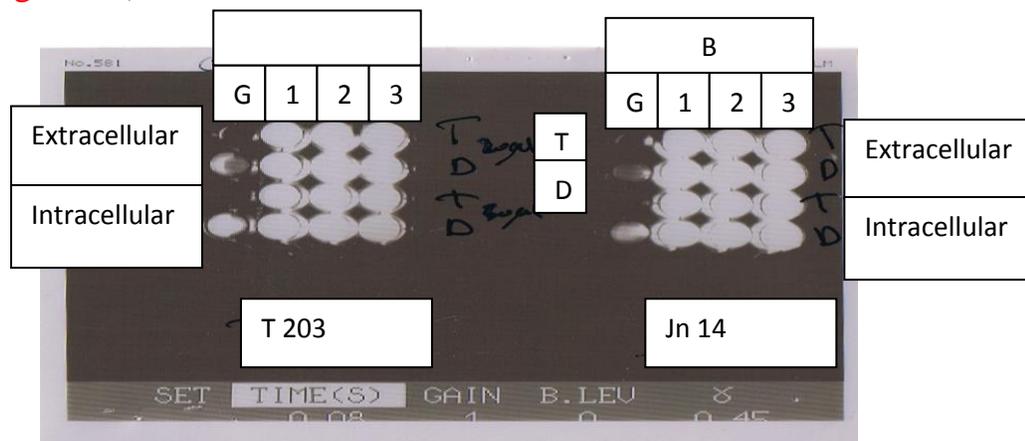


**Figure 12.** A: Endo and Exo -chitinases produced by *T. harzianum* (Jn14) and *T. asperillum* (T 203). B: Glucanases produced by *T. harzianum* isolates Jn14 and Jn58.

The differences in the mean values among the treatment groups are not great enough to exclude the possibility of random sampling effect.

### 3.4.1.1 Enzymatic assay on microtiter plate using fluorescent substrate

*T. harzianum* Jn14 and *T. asperillum* (T 203) were tested for the chitinolytic activity on microtiter plates using the substrate 4-methylumbelliferyl-N-acetyl- $\beta$ -Dglucosaminide[4-MU-(GlcNAc)] for dimeric activity, and 4-methylumbelliferyl- $\beta$ -D-N-N'-diacetylchitobioside[4-MU-(GlcNAc)<sub>2</sub>] for trimeric activity. Hydrolysis of this  $\beta$ -1, 4 linkage of 4-methylumbellifery group to the N-acetylglucosamine oligosaccharides yields the fluorescent product 4-methylumbelliferone visualized under UV. The strains were tested for extra and intra cellular protein activity after induction with colloidal chitin (Figure 13).

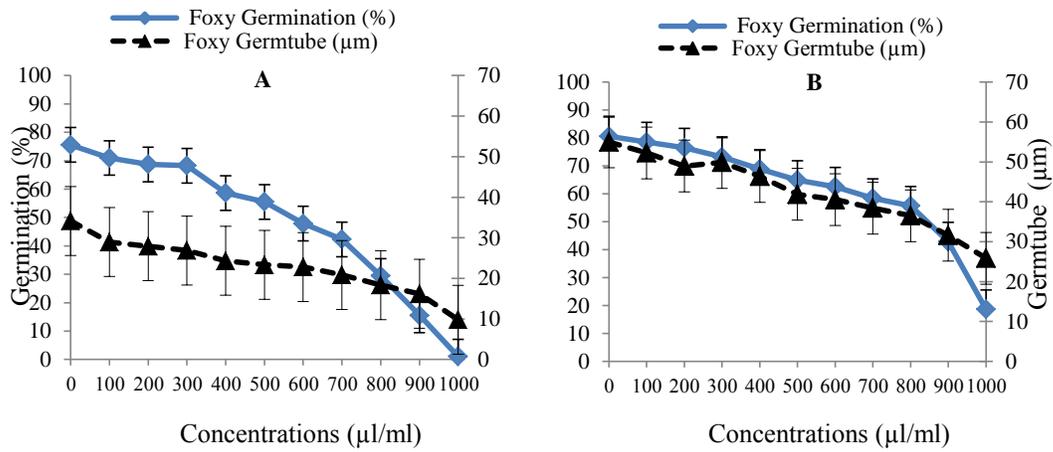


**Figure 13.** Enzymatic test on microtiter plate showing fluorescence activity under UV with both dimer and trimer substrate. (A) *T. asperillum* T 203. (B) *T. harzianum* Jn14.

The microtiter plate assay using Dimer and Trimer substrates revealed a sound activity for both species *T. asperillum* (T 203), and *T. harzianum* (Jn14) in colloidal chitin induction.

### **3.4.2 Effect of *T. harzianum* (Jn 14) enzymes (chitinases and glucanases) supernatant on *F. oxysporum* f.sp. *Lycopersici* (Lo7) germination and germ tube growth.**

The effect of *T. harzianum* (Jn 14) enzymes (chitinases and glucanases) supernatant on conidia germination (%), and germtube length of *F. oxysporum* f. sp. *lycopersici* (Lo7) was investigated (Figure 14). Results indicated that both chitinases and glucanases reduced conidial germination as well as germtube length with increasing concentrations compared to the control. Both Chitinases and glucanases completely inhibited conidial germination and germ tube elongation of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) at the highest concentration (1000 µl). Germination rates decreased proportionally at all Chitinases concentrations reaching its lowest (1.3%) at the highest concentration (1000 µl), compared with control (76%). The germ tube growth of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) decreased by (29%) at the highest concentration of chitinases compared with the control. Concerning glucanases, results showed that glucanases significantly decreased germination rates of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) conidia (23%) at the concentration (1000 µl) compared with the control (81%). The germ tube growth of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) was decreased by (47%) at the highest concentration of glucanases, compared with control.



**Figure 14.** The effects of chitinases (A); and glucanases (B) on conidial germination (%) and germtube length (µm) of *F. oxysporum* (Foxy Lo7).

[A: chitinases, Germination: **LSD= 6.077**, Germtube elongation: **LSD= 8.498**]; and [B: glucanases, Germination: **LSD= 6.486**, Germtube elongation: **LSD= 6.954**]

#### 4. DISCUSSION

The potential of *T. harzianum* as biocontrol agents against various plant diseases has been reported by several researchers (Sharon *et al.*, 2001). It is very well known, however, that biocontrol agents generally do not perform well enough under field conditions to compete with chemical fungicides. In order to enhance the antagonistic activity of *Trichoderma*, some authors have evaluated the combined effects of the antagonists with practices such as soil fumigation, solarization and fungicide application (Conway *et al.*, 1996, Elad and Chet, 1980, Khattabi *et al.*, 2001). However, few studies have been devoted to evaluating the combined effects of *Trichoderma* and nutritional factors. Matti and Sen, (1985) reported the synergistic action of some fertilizers and *Trichoderma* on *S. rolfsii*. More recently, Bulluck and Ristaino, (2002) found that organic amendments reduced the incidence of the disease caused by *Sclerotium. rolfsii* and favored the proliferation of the antagonistic micro-flora of the soil, especially *Trichoderma* species. The work presented in this study describes the ability of different nutrient sources in enhancing the potential of the biocontrol agent *T. harzianum* (Jn14), and improving its ability to control the pathogen *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) the causal fungus of tomato Fusarium wilt. The effect of exogenously applied nutrients which can enhance germination and growth of *T. harzianum* (Jn14) was tested using 3 sugars, 2 nitrogen forms,

and various cations. The three sugars (glucose, fructose and sucrose) stimulated germination and germ tube growth of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) but in different proportions and according to the concentration used.

The requirement of an external source of nutrients for spore germination by *Trichoderma* has been recorded by [Danielson and Davey \(1973b\)](#). However, the mechanism of sugar sensing by *F. oxysporum* f.sp. *lycopersici* (Lo7) conidia, is unknown. As diverse sugars and acetate induce germination with similar efficiency, it appears unlikely that nutrient sensing occurs by plasma membrane proteins ([Forsberg and Ljungdahl, 2001](#)). One explanation for the particular important activity of fructose in conidial germination could be that this sugar is preferentially taken up by a fructose specific transport system. This is surprising since glucose is usually the most efficient hexose not only as a nutrient, but also as a signaling compound ([Doehlemann et al., 2005](#)). In addition, [Monga, \(2001\)](#) reported that *T. harzianum* showed excellent sporulation on sucrose and glucose respectively, but fructose was the best carbon source for the biomass production of *T. viride* and *G. virens*. However, *T. harzianum* produced maximum biomass on maltose and glucose, respectively.

Concerning the important of inorganic nitrogen forms in vitro,  $\text{NH}_4\text{-N}$  enhanced conidia germination of *T. harzianum* (Jn14) significantly where it peaked at 100 mM (93%) compared with the control (54%) with no significant differences noticed between *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Lo7). However,  $\text{NO}_3\text{-N}$  enhanced germination of *T. harzianum* (Jn14) conidia at the concentrations below 1mM (93%). Germination rates started to decrease proportionally, however, above this concentration. On the other hand, the addition of  $\text{NO}_3\text{-N}$  has negatively influenced the germination of *F. oxysporum* f.sp. *lycopersici* (Lo7) conidia at all concentrations compared with the control (73%). Concerning the effect of nitrogen forms on the population of *T. harzianum* and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) in soil, the results showed that data almost followed the same trend where  $\text{NH}_4$  increased the population of both fungi in soil, while the addition of  $\text{NO}_3$  increased significantly the population of *T. harzianum* (Jn14) at all concentrations and decreased the population of Foxy. Disease severity results showed that the two nitrogen forms have not influenced the biocontrol efficiency of *T. harzianum* (Jn 14). Furthermore, the results showed that tomato plants growth parameters d followed the same trend. The addition of  $\text{NH}_4$  and  $\text{NO}_3$  has significantly increased tomato plant parameters in the presence of the two fungi. This was reflected positively on

plant heights and fresh and dry weights. In addition, growth parameters increased with the increase of nitrogen forms concentrations. Similarly, **Huber and Watson, (1974)** discovered that  $\text{NH}_4$  increased the germination of the pathogen, while  $\text{NO}_3$  decreased the germination of *F. oxysporum*. **Nol and Henis, (1987)** showed that the addition of ammonium nitrate to the water agar medium allowed only 25% of *T. hamatum* to germinate at  $10^9$  as compared to 78% at  $10^7$  spores. **Mekala et al., (2008)** showed that  $(\text{NH}_4)_2\text{SO}_4$  provided both the ammonium as well as sulfate ions for the conidial germination and enzyme production of *T. reesie*. **Khattabi et al, (2004)** investigated the effect of urea, sulfate ammonium; nitrate potassium on *S. rolfsii* in vitro, alone and in combination with *T. harzianum*. Results showed that *S. rolfsii* did not utilize urea as a source of nitrogen while sulfate ammonium and nitrate potassium allowed the growth of the fungus in vitro. **Mehta et al, (2012)** reported that *T. viride* showed higher growth rate and sporulation on almost all the nutrient sources tested (nitrogen sources used are Sodium Nitrate, Potassium Nitrate and Ammonium Sulphate). Within these nitrogen sources, *T. viride* showed the highest biomass production in the Ammonium Sulphate form in vitro. However, Investigations have shown that *F. oxysporum* requires 12 nutrients and an organic source of energy for normal growth in soil (**Woltz and Jones 1981**).

These are absolute requirements which, if unsatisfied, will limit growth, sporulation, pathogenicity, and survival of the fungus in soil (Smith and Snyder 1975). Several potentiall-pathogenic *Fusarium* species survive in soil as inactive resting structures called chlamyospores (Guerra and Anderson 1985). Chlamyospores require exogenous sources of carbon and nitrogen to germinate (Davey *et al.* 1996), especially at higher spore concentrations (Griffen 1970b). Ammonium may stimulate chlamyospore germination more than nitrate sources (Loffler *et al.* 1986b). Nitrogen deficiencies may inhibit chlamyospore maturation and stimulate spore lysis (Griffin, 1976). Bulluck and Ristaino, (2002) found that some organic amendments, such as swine manure, increased the population of *Trichoderma* in comparison with some mineral nitrogen fertilizers in soil. The combined use of *T. harzianum* and manure has synergetic action that affected the sclerotial viability of *S. rolfsii*. The added manure provided the carbon and the nitrogen to *T. harzianum*. Thus, it enhanced its development and its antagonistic activity. It was reported that soil organic amendments increased the multiplication capacity of *Trichoderma* (Kok *et al.*, 1996). In the same direction, Khattabi *et al.*, (2004) showed that the antagonistic activity of *T. harzianum* against *S. rolfsii* was stimulated in the presence of the three nitrogen sources. High NO<sub>3</sub> nitrogen decreased diseases caused by *Fusarium oxysporum*, *Botrytis*

*cinerea*, *Rhizoctonia solani* and *Pythium* spp. In contrast, high  $\text{NH}_4$  nitrogen decreased diseases caused by *Pyricularia*, *Thielaviopsis basicola*, *Sclerotium rolfsii* and *Gibberella zeae* (Huber and Graham, 1999; Celar, 2003; Harrison and Shew, 2001). Morgan and Timmer, (1984) showed that disease severity and pathogen propagule densities of *Fusarium oxysporum* f.sp. *citri* were greater and pH was lower in potting media fertilized with  $\text{NH}_4\text{-N}$  than in media fertilized with  $\text{NO}_3\text{-N}$ .

Concerning the effect of cations on conidial germination and germ tube growth of *F. oxysporum* f.sp. *lycopersici* (Lo7), and *T. harzianum* (Jn14) in vitro, results showed that  $\text{K}^+$  decreased conidia germination of *T. harzianum* (Jn14) and Foxy proportionally with increasing cation concentration. Concerning  $\text{Ca}^{2+}$  and  $\text{P}^+$ , results showed that  $\text{Ca}^{2+}$  and  $\text{P}^+$  increased significantly germination rates of *T. harzianum* (Jn14) conidia at the concentrations below 1 and 10 mM, respectively. On the other hand, the addition of  $\text{Ca}^{2+}$  and  $\text{P}^+$  has negatively influenced the germination of Foxy conidia at all concentrations. Furthermore, the results showed that the effect of the cations ( $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{P}^+$ ,  $\text{Fe}^{2+}$ ) on *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) population in soil followed the same trend.  $\text{K}^+$  decreased the fungal population of both fungi proportionally with the increasing cation concentration. However,  $\text{Ca}^{2+}$ ,  $\text{P}^+$ ,  $\text{Fe}^{2+}$ , increased the

fungal population of *T. harzianum* (Jn14) in the soil in large numbers and decreased the population of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) significantly compared with the control. Disease severity showed that the 4 cations have not influenced the biocontrol efficiency of *T. harzianum* (Jn 14). These results are in line with those of previous studies. [Dłużniewska, \(2003\)](#), [Kredics et al.,\(2003\)](#), and [Badura and Piotrowska-Seget, \(2000\)](#) mentioned that several heavy metal ions are trace elements necessary for the growth of fungi; at high concentrations they are toxic. The adverse effect of metals upon the growth and activity of microorganisms may result from the fact that metals can bind to various biomolecules by covalent bonds. Metals may also unspecifically affect many cell structures and influence metabolic processes through a blockage of enzymes. [Ortega-Aguilar et al., \(2011\)](#) showed that KHCO had negative effects on the growth and spore germination of both *Sclerotium cepivorum* and *Trichoderma* sp. strain R39 in vitro. [Arslan et al., \(2009\)](#) reported that mycelial growth and spore germination of *F. oxysporum* f.sp. *melonis* was totally inhibited by potassium benzoate, potassium sorbate and sodium benzoate, but not by sodium carbonate, sodium bicarbonate, or sodium citrate dihydrate. [Mills et al., \(2004\)](#) determined that mycelial growth and spore germination of *Alternaria alternata*, *Botrytis cinerea*, *Fusarium solani* var. *coeruleum*,

*Phytophthora erythroseptica*, *P. infestans*, *Verticillium albo-atrum*, and *V. dahlia* were strongly limited by potassium sorbate, sodium metabisulfite and propyl-paraben in soil. Löffler and Schippers, (2011) mentioned that KCl, and CaCl<sub>2</sub> buffer solutions at higher concentrations inhibited chlamydospore production in germinated macroconidia of *Fusarium oxysporum* in soil. In the same constant, Potassium deficiency has been found to be linked to diseases in a number of temperate crops (Palti, 1981). High K<sup>+</sup> supply can improve resistance of plants to fungal and bacterial pathogens (Marschner, 1995). High levels of potassium fertilizer have been found to reduce disease severity, but these effects seem to be related to the balance between potassium and nitrogen in soil (Woltz and Jones 1981). Under normal crop production, adequate levels of both nitrogen and potassium are required; excessive rates of nitrogen should be avoided (Woltz and Jones 1981). Jinghua *et al.*, (2004) studied the effects of potassium, calcium, and iron on the efficiency of *Trichoderma* strain T23 in controlling *Fusarium spp.* However, Türkkan, (2013) revealed that (calcium acetate, calcium chloride, diammonium phosphate, potassium bicarbonate, sodium acetate trihydrate and sodium phosphate dibasic) inhibited the mycelial growth and spore germination of *F. oxysporum* f.sp. *cepae* in vitro. Abdel-Kader *et al.*, (2012) found that calcium chloride reduced mycelial growth and spore germination

of *F. solani* and *F. oxysporum* in soil. Another study demonstrated that a lower rate of germination of *F. oxysporum* chlamydospores occurred in soils that had an adequate supply of  $\text{Ca}^{+2}$ , particularly in relation to other basic cations,  $\text{Mg}^{+2}$  and  $\text{K}^{+}$  (Chuang 1991). Woltz and Jones 1981 indicated that higher soil pH values and populations of actinomycetes also decreased spore germination rates. However, relatively low levels of calcium appear more conducive to Fusarium diseases than normal levels. An adequate supply of calcium ( $\text{Ca}^{+2}$ ) has been demonstrated to enhance resistance to a number of diseases in annual crop species caused by pathogens such as *Rhizoctonia solani*, *Sclerotium* spp., *Botrytis* spp., *F. oxysporum* and the nematodes *Meloidogyne* spp. and *Pratylenchus* sp. (Agrios 2005). Increased calcium supply to the plant has been shown to reduce the severity of Fusarium wilt of tomato (Walker, 1972). Cations competitive with calcium, such as sodium, adversely affect disease resistance in many host plants (Standaert *et al.* 1973). Calcium nitrate as well, has been shown to suppress disease (Elmer, 1989). Jinghua *et al.*, (2004) noted that soil treatment with *Trichoderma* in combination with ammonium molybdate, manganese sulphate and calcium sulphate significantly reduced melon wilt disease index. Furthermore, Mecteau *et al.*, (2008) reported that trisodium phosphate completely inhibited mycelial growth and spore germination of *F. sambicinum* and *F.*

*solani* var. *coereuleum* at 0.2 M (7.6%) in vitro. Variable effect of mineral nutrition on *Trichoderma* spp. was also observed in other studies. It was demonstrated that zinc apparently inhibited mycelial growth, whereas manganese ions stimulated *T. viride* spore germination (Sierota Z, 1982). Phosphorus nutrition improves crop vigor and may decrease severity of diseases through new growth (Buresh, 1997). Improved root growth by P<sup>+</sup> nutrition may allow the plant to ‘escape’ attack by soil-borne fungal pathogens or nematodes (Prabhu *et al.*, 2007). In general, balanced nutrition of nitrogen, phosphorus, and potassium often results in less disease caused by *Fusarium* spp. (Walker, 1946). Furthermore, Jinghua *et al.*, (2004) showed that ferrous sulphate, calcium sulphate and potassium dihydrogen improved the spore germination of *T. harzianum* T23. However, Helbig and Carroll, (1984) showed that Fe<sup>+2</sup> reduced the conidial germination of *F. oxysporum*. A critical amount of iron is required for chlamydospore production by *F. oxysporum* in soil (Simeoni *et al.* 1987). However, Guerra and Anderson (1985) found that iron and boron deficiencies resulted in greater virulence by *F. solani*.

Concerning the ability of *T. harzianum* (Jn14) to induce increased growth response in tomato plants (plant height, number of flowers, fresh and dry weights, and root fresh and dry weights), results showed that there was a general increase in the values of growth parameters of tomato plants grown in soil amended with *T. harzianum* (Jn14) compared to the non-treated control. Increased growth response of several plants including vegetables, following the application of *Trichoderma* to pathogen-free soil has been documented (Baker, 1989; Chang *et al.*, 1986; Kleifeld and Chet, 1992). In this study, *T. harzianum* (Jn14) have enhanced tomato plant heights, and flowering, as well as fresh and dry weights. These results were similar to the results revealed by (Yedidia *et al.*, 2001) who showed that treatment of cucumber plants in soil with *T. harzianum* (T-203) resulted in large increase in root area and cumulative root lengths, and significant increase in dry weight, shoot length and leaf area over that of the untreated control. Yedidia *et al.* (2001) suggested a direct role for *T. harzianum* in mineral uptake by the plant at a very early stage of the fungal plant association. In addition, Harman, (2000) established that *Trichoderma* spp. are opportunistic plant colonizers that affect plant growth by promoting abundant and healthy plant roots, possibly via the production or control of plant hormones. Increased growth response has been demonstrated as well, by several other

investigators (Altomare *et al.*, 1999; Anusuya and Jayarajan, 1998). Who demonstrated the ability of *T. viride* and *T. harzianum* to solubilize insoluble tricalcium phosphate in vitro. The concept of adding biocontrol agents into a planting mix or applying directly to the roots of transplants is an efficient, inexpensive means to provide a more vigorous transplant with disease protection when it is transplanted to the field (Nemec *et al.*, 1996).

Concerning the enzymes production (Chitinases and Glucanases) by *T. harzianum* (Jn 14) and *T. asperillum* (T 203), results showed that the production of endochitinases by *T. harzianum* (Jn14) was more than that of *T. asperillum* (T 203). However, the production of exochitinases by *T. asperillum* (T 203) was more than that of *T. harzianum* (Jn14). Concerning glucanases production, *T. harzianum* (Jn14) was better than *T. harzianum* (Jn58) in producing glucanases. Results of this study are consistent with other reports (Schlumbaum *et al.*, 1986; Mauch, *et al.*, 1988; Lorito *et al.*, 1993). It has been reported by Lorito *et al.* (1993) that since the antifungal activity of the purified chitinolytic enzymes was roughly proportional to the quantity of chitin in the cell wall of test fungi, the ability of these proteins to inhibit the fungal growth is probably a consequence of their chitinolytic activity, not of secondary toxic properties. Furthermore, Benhamou and

Chet, (1993) and Lorito *et al.*, (1993) showed that the purified endochitinases, from *T. harzianum*, which play an antagonistic and a nutritional role, is a strong inhibitor of many important plant pathogens and able to lyse not only the “soft” structure at the hyphal tip but also the “hard” chitin wall of mature hyphae, conidia, and chlamydospores. However, the antifungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes are used together (Lorito *et al.*, 1994) or in combination with other compounds (Collinge *et al.*, 1993; Sahai and Manocha, 1993). The level of inhibition for *Trichoderma* chitinases were usually higher than those described for plant, bacteria or other fungal chitinolytic enzymes assayed under similar conditions (Lorito *et al.*, 1996b). Sandhya (2004) studied the extra-cellular chitinase production by the chitinolytic fungus *Trichoderma harzianum* TUBF 966 using submerged fermentation. Maximum chitinases production (14.7 U/ml) was obtained when fermentation was carried out at 30°C for 96 hr using 72 h old mycelium in a medium containing colloidal chitin 1.5% (w/v) as carbon source. Furthermore, Production of extracellular  $\beta$ -1, 3-glucanases, chitinases, and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (De la Cruz *et al.*, 1995). These observations,

together with the fact that chitin,  $\beta$ -1,3 glucan and protein are the main structural components of most fungal cell walls (Peberdy, 1990), are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens (Chet and Baker, 1981). *Trichoderma* spp. (especially *T. harzianum* and *T. viride*) exhibit considerable variability among strains with respect to their biocontrol activity and host range (Sivan and Chet, 1992). Thus, new strains, which are more effective in biocontrol, could have considerable industrial potential. Screening twenty-four *Trichoderma* isolates a strain identified as *T. harzianum* Rifai was found to be the best producer of chitinases and  $\beta$ -1, 3-glucanases. Furthermore, Garcidueñas *et al.*, 1998 showed that the biocontrol agent *Trichoderma harzianum* IMI206040 secretes  $\beta$ -1, 3-glucanases in the presence of different glucose polymers and fungal cell walls. The level of  $\beta$ -1, 3-glucanase activity secreted was found to be proportional to the amount of glucan present in the inducer. The fungus produced at least seven extracellular  $\beta$ -1, 3-glucanases upon induction with laminarin, a soluble  $\beta$ -1, 3-glucan.

Concerning the effect of *T. harzianum* (Jn 14) enzymes (chitinases and glucanases) supernatant on conidia germination (%), and germ tube length of *F. oxysporum* f. sp. *Lycopersici* (Lo7) results showed that both chitinases and glucanases reduced conidial germination as well as germ tube length with increasing concentrations compared to the control. Both Chitinases and glucanases completely inhibited conidial germination and germ tube elongation of *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) at the highest concentration (1000 µl). Similarly, [Viterbo et al., 2001](#) mentioned that culture filtrates containing secreted CHIT36 as the sole chitinolytic enzyme completely inhibited the germination of *Botrytis cinerea* conidia. Growth of *Fusarium oxysporum* f. sp. *melonis* and *Sclerotium rolfsii* were significantly inhibited on agar plates on which the *Trichoderma* had previously been grown.

**In conclusion**, some exogenously applied nutrients (e.g. NO<sub>3</sub>-N, Ca<sup>2+</sup>, P<sup>+</sup> and Fe<sup>2+</sup>) can enhance the growth and proliferation of *T. harzianum* (Jn14) in soil and hence might improve the antagonistic efficiency of the bioagent against *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) who showed negative reaction in general towards these nutrients. This however, needs further studies to elucidate the way these elements function in supporting *T. harzianum* (Jn14) against soil borne pathogens, and to expand the trials to

include field application at a wider scale. In addition, this study showed that *T. harzianum* (Jn14) proved to produce considerable amounts of chitinases and glucanases very comparable to world well-known isolates of this fungus. However, further studies are still needed for shedding more light on the antibiosis capacity of *T. harzianum* (Jn14).

زيادة فعالية مكافحة الحيوية باستخدام عزلة محلية من فطر التريكوديرما في مكافحة مرض الذبول الفيوزارمي على نباتات البندورة.

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## الملخص

يعتبر مرض الذبول الفيوزارمي على البندورة والذي يسببه *Fusarium oxysporum* f.sp. *lycopersici* (Foxy Lo7) من أهم الامراض المحددة للإنتاج في جميع مناطق زراعة البندورة في العالم.

تهدف هذه الدراسة الى فحص إمكانية زيادة فعالية مكافحة الحيوية لعزلة التريكوديرما المحلية (Jn14) في مكافحة مرض الذبول الفيوزارمي على نبات البندورة. خلال هذه الدراسة تم فحص مقدرة عدد من العناصر الغذائية ( السكريات وأنواع النيتروجين والكاتيونات) في زيادة فعالية العزلة المحلية من فطر التريكوديرما *Trichoderma harzianum* (Jn14) في مكافحة مرض الذبول الفيوزارمي على نبات البندورة داخل المختبر والبيت الزجاجي. أظهرت النتائج ان اضافة النيترات والكالسيوم والفسفور عملت على زيادة النمو ونسب إنبات الأبواغ لفطر التريكوديرما *T. harzianum* (Jn14) بنسب عالية، في حين قللت من نمو ونسب إنبات الأبواغ لفطر الفيوزاريوم *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) في المختبر. كما أظهرت النتائج ان إضافة النيترات والكالسيوم والفسفور والحديد عملت على زيادة أعداد فطر التريكوديرما *T. harzianum* (Jn14) في التربة، في حين قللت من أعداد فطر الفيوزاريوم *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) في التربة. بالإضافة الى ذلك، أثبتت عزلة التريكوديرما قدرتها على تحسين مقاييس نمو نبات البندورة داخل البيت الزجاجي .

ومن جهة اخرى، أثبتت الدراسة ان فطر تريكوديرما بعزلته المحلية *T. harzianum* (Jn14) لديها قدرة عالية لإنتاج أنزيم endochitinases أعلى من تلك الخاصة بفطر التريكوديرما *T.*

*T. asperillum* (T 203) بـ 15% ، في حين أنتج فطر التريكوديرما (*T. asperillum* (T203) exochitinases بسنة أعلى من فطر التريكوديرما (*T. harzianum* (Jn14) بـ 2%. بالإضافة الى ذلك أثبتت عزلة تريكوديرما (*T. harzianum* (Jn14) قدرتها على إنتاج انزيم الغلوكوناز بنسبة أعلى من عزلة تريكوديرما (*T. harzianum* (Jn58) بـ 21%. كما أشارت الدراسة الى أن نواتج أيض فطر التريكوديرما (*T. harzianum* (Jn14) أثبتت قدرتها على تثبيط نمو ونسب إنبات أبواغ فطر الفيوزاريوم (*F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) على أعلى تركيز تم فحصه (1000 µl/ml) في المختبر.

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## List of Appendices

### Appendix 1. Fusarium disease severity



Tomato plant grown in soil inoculated with Foxy and Jn14, amended with different nutrient concentration)



CK (tomato plant grown in soil without inoculum with fungi)



Tomato plant grown in soil inoculated with Foxy)

Appendix 2. Effect of nitrogen forms ( $\text{NH}_4$  and  $\text{NO}_3$ ) in the presence of *T. harzianum* (Jn14) and *F. oxysporum* f.sp. *lycopersici* (Foxy Lo7) on tomato plant growth parameters.



Appendix 3. Increased growth response of tomato plants induced by *T. harzianum* (Jn14).



## Appendix 4. ANOVA Tables

Experiment	DF treat.	DF resid.	DF total	SS treat.	SS resid.	SS total	MS treat.	MS resid.	F
Effect of NO <sub>3</sub> added to Jn14 and Foxy on height of tomato plants	3	28	31	4353.250	350.75	4704	1451.083	12.527	<0.001
Effect of Ca concentrations on <i>T. harzianum</i> population in infested soil	3	92	95	1129.750	1089.583	2219.333	376.583	11.843	<0.001
Effect of Fe concentration +Jn14 and Foxy in the soil on height of tomato plants	3	28	31	620.625	1594.250	2169.875	206.875	55.440	<0.001
Effect of Ca concentration +Jn14 and Foxy in the soil on height of tomato plants	3	28	31	282.125	535.750	817.875	94.042	19.114	<0.001
Effect of P concentration +Jn14 and Foxy in the soil on height of tomato plants	3	28	31	11.094	566.875	577.969	3.698	20.246	<0.001
Effect of NH <sub>4</sub> concentration +Jn14 and Foxy in the soil on height of tomato plants	3	28	31	2228.594	1277.875	3506.469	742.865	45/638	<0.001
Increased growth response of tomato plants	3	28	31	2796.844	2912.625	5709.469	932/281	104.022	<0.001
Effect of Glucose on conidial germination of <i>Trichoderma</i> Jn14.	5	138	143	26981	52840	79821	5396	382	<0.001

Effect of chitinases concentrations on conidial germination of Foxy after 20 hours.	10	253	263	142269	28910	171179	14226	114	<0.001
Effect of glucanases concentrations on conidial germination of Foxy after 20 hours.	10	253	263	79487	32930	112418	7948	130	<0.001
Effect of endochitinase amount between T203 and Jn14.	1	10	11	1045	12089	13135	1045	1208	<0.001

بسم الله الرحمن الرحيم



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