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Effect of Environmental and Host Factors on Biological Control of Fusarium wilt by Non-pathogenic *Fusarium oxysporum* in Tomato

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Abstract

This study was focused on the effect of environmental and host factors on the antagonism of Fusarium wilt of tomato, caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol), by non-pathogenic strains of *F. oxysporum*. Seven non-pathogenic strains of *F. oxysporum* were isolated and screened for antagonism of Fusarium wilt. Strains F1 and F4 were chosen for further experiments as they reduced disease severity more than other non-pathogens. The best method for applying non-pathogens and pathogens was to inoculate soil with conidial suspension. The non-pathogens reduced seed germination and growth of tomato plants in the absence of the pathogen. In a split root system, non-pathogens F1 and F4 induced resistance of tomato plant against Fusarium wilt although there was no direct contact between the pathogen and non-pathogens. Iron at high and standard concentration in the nutrient solution stimulated induced resistance. However, direct antagonism of Fol by F4 was greatest at low level of iron.

Tomato root exudates increased in the vitro antifungal activity of non-pathogens toward pathogens and also increased spore germination of both non-pathogens and pathogens. The components of root exudates including sugars and organic acids influenced the antagonism of non-pathogens against pathogen in vitro. However sugars and organic acids had little effect on disease suppression in pot trials.

In dual culture, using $NaNO_3$ as source of N, the inhibition of Fol by F1 and F4 was decreased at high level of N, whereas at high level of $NaNO_3$ as source of N the antibiotic production increased. Using NH_4Cl as source of N at high and low level, the inhibition of Fol by F1 and F4 was increased and the antibiotic production of non-pathogens also increased. In pot trials, the disease severity was less at low N compared with high level of N.

In dual culture, at low level of K the antagonistic activity of s F1 and F4 against Fol was improved. However, at high level of K, the antibiotic production of non-pathogens increased. In glasshouse pot trials, non-pathogens improved plant health at low level of K, whereas the growth of non-pathogens was decreased at high level of K.

At high level of Ca, the inhibition of growth of Fol by F1 and F4 was decreased. Also at low level of Ca the inhibition of growth of Fol by antibiotic production of F4 was increased.

However, the inhibition of growth of Fol by antibiotic production of F1 was increased at high level of K.

The inhibition of growth of Fol by F1 and F4 was decreased with high level of iron. The antibiotic production of F1 and F4 inhibited growth of Fol at low level of iron more than at high level. Biological control did not work well at high levels of iron.

Further work is needed on the effect of non-pathogens on the growth of plants. More tests should be done on the effect of root exudate on antagonism. Biocontrol agents should be found that can work well at low nutrient levels.

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Certification

I certify that the ideas, analyses, results, and conclusions reported in this thesis are entirely my own effort, except where otherwise acknowledged. This work has not previously been submitted for a degree or diploma in any university.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signature of Candidate  Date 05/ 12/ 2017

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Chapter 1. General Introduction

Tomato *(Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) is an important crop that is cultivated widely in the world. Tomato has its origin in the South American Andes (Naika et al., 2005). Tomato plants are infested by several diseases caused by bacteria, viruses and fungi. Tomato wilt diseases are caused by several pathogenic fungi including *Verticillium* and *Fusarium* species. *Fusarium oxysporum* is a soilborne fungal pathogen which has the ability to infect plants at all stages of plant growth through roots, causing necrosis and wilting symptoms in many crop plants which leads to major economic losses (El- Khallal, 2007). *F. oxysporum* f. sp. *lycopersici* (Fol) is the cause of Fusarium wilt of tomato which is considered a major restrictive factor in the production of tomato (Ignjatov et al., 2012). It causes serious damage to both field and greenhouse-grown tomato plants. The main evidence of Fusarium wilt is browning of the vascular system (Wong, 2003).

Controlling this pathogen by rotational cropping is not useful, because the pathogen is soilborne and it has the ability to persist in the soil for a long period time without a host. Also infected dead plants cause the spread of the pathogen, so different control methods should be used for *F. oxysporum* (Ignjatov et al., 2012; Validov et al., 2007).

Biological control of soilborne plant pathogens can be achieved by application of natural antagonists of the pathogen. Using Fusarium-resistant tomato plant cultivars could provide protection against this disease, however new races of the pathogen can defeat resistance to the cultivar which creates a problem. Using methyl bromide fumigation was the most effective method to control Fusarium wilt of tomato. However methyl bromide was outlawed in 2005 because it caused serious environmental problems. Many antagonists used as biological control reduced the incidence of Fusarium wilt of tomato, such as *Penicillium oxalicum* (Cal, 1999). Most of the microorganisms have the ability to consume nutrients secreted by plants and occupy niches on the root surface faster than the pathogen which leads to the competition between them (Compant et al., 2005). Nonpathogenic strains of *F. oxysporum* have the ability to induce suppressiveness to pathogenic strains in the soil and play an important role as biological control in the soil. Biological control of soilborne plant pathogens can occur by competition for nutrient, primarily carbon, nitrogen, and iron (Benson and Baker, 1970; Cook and Schroth, 1965). However, the activity of biological control differs depending on the culture conditions of tomato plants.

Induced resistance is one of the factors in biological disease control. Fuchs et al. (1997) reported that in split root systems, the non-pathogen *F. oxysporum* strain Fo47 induced resistance in tomato plants against pathogenic Fol by increasing chitinase and β-1,4-glucosidase activity in the plant. *Pseudomonas fluorescens* WCS417r and non-pathogen Fo47 had the ability to induce resistance of tomato plants possibly by accumulation of chitinases against Fusarium wilt (Duijff et al., 1998). The disease severity of *Fusarium oxysporum* f. sp. *asparagi* was reduced for *Asparagus officinalis* plants treated with non-pathogenic *F. oxysporum* (npFo) compared with plants untreated with the non-pathogen (He et al., 2002).

Also, understanding the role of root exudate is very important to control disease. Kravchenko et al. (2003) reported that root exudate of tomato plant plays important role in the growth and antifungal activity of plant growth-promoting *Pseudomonas* strains showing that the antifungal activity of plant growth-promoting rhizobacteria in the rhizosphere could depend on the composition of root exudates such as sugar and organic acid. There was a relationship between usage of sugar and amino acids in the root exudate and the diazotrophic population, while the root exudate from different rice cultivars may have different effects on the population of diazotrophs (Naher et al., 2009).

Nutrients also affect the biological control of diseases. The competition for Fe is able to induce suppressiveness to Fusarium wilt pathogens (Scher and Baker, 1982). At low concentration of iron, strains WCS374 of *P. fluorescens* and its pseudobactin-minus mutants gave higher protection than at high concentration of iron against Fusarium wilt of radish caused by *Fusarium oxysporum* f. sp. *raphani* (Leeman et al., 1996). At 10mM Fe the effect of *Trichoderma asperellum* strain T34 on disease severity of Fol on tomato plants was greater than at 1000mM Fe (Segarra et al., 2010).

Jabnoun-Khiareddine et al. (2016) found that potassium sorbate had antifungal activity on fungal tomato pathogens Fol, *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and *Verticillium dahliae* (VD) as a direct effect, and indirect effect by decreasing tomato wilt severity incited by Fol, FORL and VD by improving growth of tomato plants. The nitrogen availability restricted the activities of antagonists of *Fusarium pseudograminearum* in straw, so the displacement of *F. pseudograminearum* from straw pieces was increased when *Trichoderma harzianum* was combined with nitrogen (Singh Lakhesar et al., 2010). Previous studies indicated the role of calcium chloride to improve the activity of strain 138 of

Kloeckera apiculata toward gray and blue molds of apple and Rhizopus rot of peach (Mclauchlin et al., 1992).

The aim of this study was to understand the role of environmental and host factors on the antagonism of Fol on tomato by non-pathogenic strains of *F. oxysporum*. Antagonistic strains of *F. oxysporum* that can reduce symptoms of Fusarium wilt of tomato were isolated and identified. Then the ability of these antagonists to induce resistance of tomato plants against the pathogen was tested. The role of root exudate of tomato plant in growth of pathogen and non-pathogen was studied and the effect of root exudate on the efficacy of antagonists against pathogen was tested. Also, the effect of major plant nutrients on the interaction of antagonists against the pathogen and on biological control was examined.

Chapter 2. Literature Review

2.1. Tomato wilt

Tomato (*S. lycopersicum*) is one of the most popular vegetables crops in the world. It contributes to a healthy, good balanced diet. Tomato is a perfect source of vitamins, and minerals such as iron and phosphorus. The tomato crop is susceptible to different sorts of diseases and disorders. There are many possible causes of tomato diseases, such as bacteria, viruses, nematodes, oomycetes and fungi. Fusarium wilt caused by the fungus Fol is considered one of the most important diseases in the world. This fungus is a soil borne pathogen that infects tomato plants at all stages of growth through the roots. This disease causes excessive losses, particularly on the susceptible varieties of tomato (Cotxarrera et al., 2002; Gilreath et al., 1994; Srinon et al., 2006). There are similarities of symptoms for many plant diseases. It is very necessary to study the symptoms and signs of pathogens to diagnose the condition of the plant and to identify the causative pathogen definitely and accurately (Bost, 2011).

2.1.1. Disease cycle

Three kinds of spores are produced by *F. oxysporum*. The colourless and slightly curved macroconidia usually have four cells. Microconidia are unicellular, small and without any colour. Chlamydospores are round, with thick walls and they can develop on or in mycelium or from cells of the macroconidia (University of Illinois Extension, 1990). The most important source of inoculum in soil is chlamydospores. Couteaudier and Alabouvette (1990b) confirmed that the percentage of germination of chlamydospores were higher than microconidia in the rhizosphere of flax seedlings. In addition with low inoculum density of the chlamydospores, it had the ability to produce more disease on flax than the microconidia.

There are several ways the fungus could transfer from soil to soil. The fungus maybe brought by seeds and transplants from wilt carrying soil, or by windborne or water spread of soil, or by garden tools, field machinery, infested tomato stakes and cages or any other way of bringing a small quantity of infested soil in the normal condition (University of Illinois Extension, 1990). Then when seedlings are transplanted in the infested soil, the fungus starts to grow in the soil adhering to the root. After that the fungus creates a small hyphal network on the root surface. Then the hyphal networks on the root surface grow denser and start to

merge. After that, the fungus appears at the base of the lateral roots and after different times the fungus was noticed penetrating epidermal cells (Olivain et al., 2006). The fungus arrived at the stele after invasion of the cortex then fungus was noticed growing from one cell to another by digestion of the cell walls. The fungus penetrates into the apex and leads to rapid destruction of the apical cells then the fungus enters the hypodermis and reaches the xylem (Olivain and Alabouvette, 1997). The fungus then grows through the xylem of roots and stems. After this, these tissues are blocked which leads to wilt of plants. Wilting can also result from fungal toxins.

The fungus may live for an indefinite length of time after its arrival, particularly in hot soils like in the greenhouse or when there are no tomato plants. It can live in the fibrous root systems of many plants including weeds like species of crabgrass and mallow (University of Illinois Extension, 1990).

2.1.2. Symptoms

Symptoms are reactions expressed by the host plant as a result of injury by the pathogen or are external or internal changes that appear on the plant after an infection by any kind of disease. Symptoms depend on the type of the pathogen and kind of the plant. The tomato plants are infected by *F. oxysporum* in greenhouses and field. It affects the plant at all stages of growth. Symptoms appear in the form of wilting and drooping of the lower leaves accompanied by the loss of the green colour of the main veins of the leaves. Often plants die before reaching the stage of flowering or fruiting. In old plants, the symptoms appear in the form of yellowing and wilting of plant. These plants may get wilt on one side and that further spreads to all parts of the plant. Thus it stops plant growth and eventually gets permanent wilt, causing change in the colour of the leaves from normal green to brown. The plants produce small amounts of fruit or zero production when attacked by the pathogen *F. oxysporum* under suitable environmental conditions in early stages of plant development (University of Illinois Extension, 1990).

There are many characteristics that could identify the disease. When the skin and bark are cut for a fraction of the main stem near the base of the plant, there will be noted discoloration (brown colour) of the vessels in the form of brown lines. In case of wet weather, one can note the pink white mycelium on the injured stem or its remains in the dead part of the plant. The spores of the pathogenic fungus badly infest these sites. Then, the affected site of the plant

speeds up the wilting and death of leaves of the plant. The seeds that are planted in the soil containing pathogenic fungus lose their ability to grow and cause significant economic losses. That is why many farmers suffer economically from this disease (University of Illinois Extension, 1990).

2.1.3. Factors that affect disease

2.1.3.1. Temperature

Larkin and Fravel (2002) claimed that the temperature has an effect on the wilt disease. They found wilt disease incidence was low (7.8%) at cool temperature of 22°C, was 77 to 82% at warm temperature of 27°C, and at high temperature of 32°C the disease incidence was 45%. Fusarium wilt occurred at a range of temperature from 20-34°C, however 27°C was the best temperature for the pathogen and wilting of the host plant happened most quickly and severely.

2.1.3.2. Light

Larkin and Fravel (2002) claimed that light condition has an effect on the disease occurrence. Plants exposed to shade had lower disease incidence, compared with full light. Foster and Walker, 1947 reported that Fusarium wilt at light of low intensity (100 ft-c) or of short duration developed quickly in the susceptible Bonny Best cultivar, but more slowly in the multiple-gene resistant Marglobe.

2.1.3.3. Soil moisture

In a susceptible cultivar wilt disease was found to be highest at a moderate moisture level (20-32% of water- holding capacity), which was most suitable for growth (Clayton, 1923). In addition, high soil moisture levels lead to high severity of wilt disease. Disease was more severe in two cultivars of plants held at nearly 89, 95, and more than 100% of water- holding capacity(Endo et al., 1975). Plants grown in soil with high moisture availability and poor aeration had high mortality, because they are considered important factors for diseases development. But, when the plant gets enough water and good aeration plants remained tolerant to the disease. Also, with approximately- saturation levels of moisture in the soil as well restricted aeration can inhibit the ability of the host to mount a successful resistance to infection (Frank and Bakker, 1975).

2.2. Biological control

2.2.1. Definitions

Biological control is defined as the use of micro-organisms or their products to resist or eliminate pathogenic micro-organisms and their effects on plants. Biological control can use the microorganisms from the environment directly, or bring about a change in their characteristics, which leads to the spread and increase in effectiveness, or it can use one of their products (Pal and Gardener, 2006).

There are various methods to control pathogens. Soil fumigants lead to almost elimination of most microbial residents, so that leads to low microbial activity in the soil which often improves pathogen activity. Therefore, this leads to increased population of pathogens which often cause more damage than those initially targeted for control (Gamliel et al., 2000). Use of resistant cultivars is one of the most acceptable and economic systems of control of pathogens. However, sometimes new races of pathogen can destroy the resistant cultivars after a few years of commercial use (Alabouvette and Couteaudier, 1992). Therefore, the main idea concentrates on using Integrated Pest Management (IPM) to control pathogens and to avoid damaging the environment. Biological control is one of the components of Integrated Pest Management programs that highly focus on the protection of plants from pathogen. So, this study will work on the biological control of Fusarium wilt. There are many possible ways for biological control to work.

2.2.2. Competition for space

One of the biological control methods depends on the ability of biocontrol agents to colonize large parts of the root system and prevent pathogens from infecting the plant. Colonization of microorganisms on the roots can be affected by several circumstances such as environmental, nutrients, plant species and others. For example, Hadar et al. (1984) tested two isolates of *Trichoderma*, *T. harzianum* T- 18 from a Columbian soil, and *T. harzianum* T-12 from New York soil against *Pythium spp*. to protect pea seeds. They found both isolates had the ability to colonize all parts of the root system, and grew well on pea seeds which led to preventing infection of seeds by pathogenic *Pythium spp*. Papavizas (1985) studied the ability of

Trichoderma spp. to control pathogens. He found *Trichoderma spp*. can exclude pathogens, such as *Fusarium spp*. because they are very fast growing and rapidly colonize substrates. Treatment of fruit of cherry tomato with *Trichoderma* spore suspension (10^6, 10^7 and 10^8 cells per ml) played an important role to decrease incidence of rot symptoms at high concentration by inhibiting pathogen spore germination on the surface of tomato fruits (El-Katatny and Emam, 2012).

There are many strains of fungi can that colonize the roots of plants without causing any evident symptoms, these strains are defined as non-pathogen parasites. On the other hand there are strains that can induce evident symptoms which may be called pathogenic (Armstrong and Armstrong, 1981). There has been a lot of work on non-pathogenic FO, especially Fo47, in the soil. *F. oxysporum* is considered one of the most common fungi in the soil. Non-pathogenic *F. oxysporum* strain Fo47 was isolated from a soil suppressive to Fusarium wilt at Châteaurenard, France (Alabouvette, 1986). Its efficacy against pathogenic Fusarium has been established in tomato and other plants (Alabouvette et al., 2009; Fravel et al., 2003). It has the ability to stay for a long time in the soil as chlamydospores, or as active saprophytes on the organic residues (Burgess, 1981) In the past, there was no way to distinguish between non-pathogenic and pathogenic strains of *F. oxysporum*. Therefore, there was just one way which was inoculation of the host plant. So, if it induced symptoms it was called pathogenic fungi or if it cannot induce symptoms it was called non-pathogenic (Katan et al., 1991; Kistler et al., 1991).

Studies in soil naturally suppressive for Fusarium wilt led to the use of non-pathogenic *Fusarium* against Fusarium wilt. These studies found most suppressive soils had a huge population of non-pathogenic Fusaria (Smith and Snyder, 1971a; Smith and Snyder, 1971b; Toussoun, 1975). On the other hand, not all *Fusarium* spp. isolated from suppressive soil had the ability to induce suppressiveness in disinfected soil. The percentage of population of the species *F. oxysporum* that caused suppression was higher than for the species *Fusarium solani* (Tamietti and Alabouvette, 1986; Tamietti and Pramotton, 1990). Previous studies refer mostly to strains of *F. oxysporum* which have the ability to restrict the incidence of Fusarium wilt in the disinfected soil, but they were not able to distinguish which strain was more active (Croman et al., 1986). There is a relationship between the population density of the non-pathogenic strain and biological control of Fusarium wilt. With increased population density of non-pathogenic Fo47, the disease severity is reduced. After these results, they

started to use non-pathogenic strains to control Fusarium wilt and choosing non-pathogenic strains which have a good efficacy by knowing the mode of action (Duijff et al., 1998; Lemanceau et al., 1993).

There is evidence that non-pathogenic strains of *F. oxysporum* are better competitors for space on the root surface than pathogens. Olivain et al. (2006) studied the interactions between non-pathogenic Fo47 and pathogenic Fo18 at the root surface of tomato. After three days from transplantation of tomato seeds, the density of colonization of *F. oxysporum* reduced from the upper part of the root towards the elongation zone. The colonization of Fo47 entered the root surface and started to form chlamydospores. Also, after 2 days when Fo47 and Fo18 were inoculated at the same concentration, Fo47 was discovered alone on younger parts of the root. The density of colonization of Fo47 was more than Fo18 on the root surface after three days of culture. Mandeel and Baker (1991) reported that *F. oxysporum* C5 when introduced into the soil prevented *F. o. cucumerinum* from colonization of the root of 4 to 5 days old cucumber plants Nahalkova et al. (2008) studied the inoculation of tomato roots at the concentration of 1 $x10^3$ and 1x 10^5 spores/ml by Fo47 and Fo18 respectively. After 5, 6 days, the hyphae of Fo47 grew, collected and produced an intense network of hyphae and it penetrated into the upper and middle part of the root, while growth of Fo18 was restricted to a few hyphae growing between the mycelial network of Fo47 in the upper part of the taproot and some of lateral roots.

Non-pathogens appear to induce less defence reaction from the host but are more susceptible to it. The pathogenic Fol can quickly colonize the root surface at 24 h after inoculation, and at this time the pathogen penetrated into the epidermis of the root. It continued growth towards the stele, although there was a barrier formed in the hypodermis and cortex by the plant which the pathogen passed to reach the xylem. However, the barrier in the cortex prevented a non-pathogenic strain from reaching to the stele (Olivain and Alabouvette, 1997).

Olivain et al. (2003) were working on flax wilt. Flax was inoculated with Fo47 and Foln3 by dipping radicles in a microconidial suspension of 10^6 microconidia / ml for 1 h. They observed that when Fo47 colonized the outer cortex of the root the plant reaction was formation of wall appositions, collapsed cells and osmiophilic material, whereas these were less frequent and less intense in the root colonized by Foln3 and the dead cells in the flax root were more with Fo47 than Foln3.

The first stage, H_2O_2 production in the flax plant was formed by germinated microconidia of the pathogenic Fo1n3. When flax plants were inoculated by Fo1n3, the Ca_{2+} influx was higher than in the control cells, whereas, it was lesser than that induced by Fo47. The germinated microconidia for non-pathogenic Fo47 and pathogenic Fo1n3 stimulated the death of the flax cells at the same rate till 12h postinoculation. However, the percentage of cell death as result of inoculation with Fo47 was higher than the percentage of cell death when inoculations with Fo1n3 after 18 h post inoculation. It was 93%, 56% respectively (Olivain et al., 2003).

Fo47 was faster than pathogen Fo18 in the saprophytic development and development of Fo47 was also greater than Fo18 after 18 h. In addition, after three days of transplantation Fo47 had more evenly colonized through the entire root surface of tomato plants and begun to form chlamydospores whereas the density of colonization for Fo18 was decreased from the upper part of the root toward the elongation zone (Olivain et al., 2006).

2.2.3. Competition for nutrients

Competition for a nutrient can be defined as indirect interaction between microorganisms. This could be especially very important where a resource is in very limited supply, and one fungus has a high demand for the resource such as, carbon, nitrogen, iron or potassium. Therefore, one of the competitors would be present more strongly than others without apparent interaction. Previous study indicated that competition for nutrient could play an important role in the biological control of *Penicillium digitatum* by *Debaryomyces hansenii* on grapefruit (Droby et al., 1989). Also, the competition for nutrient in greenhouse between the pathogen of Pythium damping off and bacteria led to reduce oospore germination in rhizospheres of wheat, tomato, cucumber, melon, bean and cotton (Elad and Chet, 1987). Khattabi et al. (2004) reported that adding fertilizer to the soil such as urea, ammonium sulphate, potassium nitrate or manure showed increased antagonistic action of *T. harzianum* against sclerotia of *Sclerotium rolfsii*.

So, each nutrient has a different effect on biological control. Competition for carbon in disinfested soil and in the nutrient solution between non-pathogenic *F. oxysporum* and pathogenic was studied by Couteaudier and Alabouvette (1990a), as one of the mechanisms of biological control. Larkin and Fravel (1999) studied the effect of non-pathogenic Fo47 and different concentrations of glucose on the germination of chlamydospores for pathogenic

Fusarium wilt for many crops. There was no effect for other non- pathogens *Fusarium spp*. CS-1 and CS-20 on the germination or germ tube growth. These results showed the ability of Fo47 to compete for the nutrient, but *Fusarium spp*. CS-1 and CS-20 had lesser or no activity for competition for nutrition. Germination of chlamydospores of *Fusarium oxysporum melonis* and *F. o. vasinfectum* was decreased by addition of pregerminated conidia of *T. harzianum* T- 35 to soil. However, the inhibition of chlamydospore germination by *T. harzianum* was abolished when soils were amended with concentrations of glucose and asparagine higher than 0.3 and 0.006 mg/g of soil respectively (Sivan and Chet, 1989). In addition, when T-35 was applied as either seed coating or a conidial suspension with addition of root exudates to soil infested with *F. o. vasinfectum* and planted with cotton, it resulted in a decrease in the effectiveness of disease control (Sivan and Chet, 1989).

Also, in presence of three nitrogen sources, the antagonism of *T. harzianum* was stimulated on solid culture media against pathogen *Sclerotium rolfsii* (Khattabi et al., 2004). Enzyme production by *Trichoderma* was affected by using nitrogen sources such as ammonium sulfate, potassium nitrate and urea (Donzelli and Harman, 2001). The viability of sclerotia of *S. rolfsii* was decreased by urea but not by calcium ammonium nitrate. Also the effects of *Trichoderma* combined with urea on the sclerotia of *S. rolfsii* was improved (Matti and Sen, 1985). However, this may not be due to direct competition for nitrogen.

The disease incidence of various experiments was reduced when nitrogen fertilizers and organic amendments were used (Hoynes et al., 1999; Jenkins and Averre, 1986). Fusarium populations were reduced by adding nitrogen to straw (Pereyra and Dill-Macky, 2004). Also, the survival of *Fusarium avenaceum* (Fries) Sacc. in oat straw was reduced by adding urea amendment only (Kollmorgen, 1974). The growth and the sporulation of *Fusarium oxysporum* f.sp. *aeidis* were good, on 10 nitrogen compounds tested including sodium, ammonium and potassium nitrates, peptone and DL-leucine (Oritsejafor, 1986). In addition, the growth of *Fusarium solani*, *F. avenaceum*, and *F. oxysporum* on an agar medium minus K salts was arrested KNO_3 and Ca (NO_3) were most favorable, whereas NH_4 and $(NH_4)_2SO_4$ inhibited growth of fungi (Korobeinikova, 1960). Previous study suggested that activities of antagonists of *F. pseudograminearum* were limited by availability of nitrogen (Singh Lakhesar et al., 2010). Growth of *Fusarium oxysporum* var. *nicotianae* was good when ammonium nitrate, potassium and calcium were used (Khilare and Ahmed 2011).

Other studies proved that organic and inorganic salts were varied effect on the microbial strains and mycelial growth of pathogens was decreased at highest concentration of salt tested (Fagundes et al., 2013; Olivier et al., 1998).

Calcium chloride played an important role to enhance the activity of strain 138 of *Kloeckera apiculate* toward gray and blue molds of apple and Rhizopus rot of peach (Mclauchlin et al., 1992).

Another study included the role of *Trichoderma asperellum* T34 to control Fusarium wilt disease by competition for iron by using four concentrations of iron. *T. asperellum* strain T34 significantly reduced the severity of Fol on the tomato plant at 10 mM Fe compared with other concentrations. Also, the population of the pathogen Fol in the growth media for tomato plants was decreased by treatment by T34 compared with using the pathogen Fol alone with all Fe concentrations. In addition, treatment of plants with T34 plus Fol gave significantly larger heights and weights than those that were treated with Fol alone. T34 played an important role to improve plant height at the optimal Fe concentration compared with control plants. Also at 1,000 mM Fe, tomato plants were significantly improved in height and dry weights when treated by T34 (Segarra et al., 2010). Growth of *Fusarium oxysporum* var. *nicotianae* was good when the micronutrients iron, zinc and copper were used (Steinberg, 1950). Also, they found that production of siderophore by bacterial antagonists can assist in competition for iron, whereas the non-pathogen *F. oxysporum* competed for carbon sources (Lemanceau et al., 1993). When composts lack available nutrients, the pathogens *Pythium* and *Phytophthora* could be suppressed by biocontrol agents existing in suppressive composts (Hoitink et al., 1991). Spore germination and germ tube elongation of *Penicillium digitatum* reduced with increasing concentration of $CaCl_2$ while combining calcium salt with *Pichia guilliermondii* enhanced control of postharvest decay (Droby et al., 1997).

2.2.4. Antibiosis

Antibiosis means inhibition of one organism by metabolites of another. These metabolites which have the ability to kill or inhibit another organism can be called toxins. Most fungi have the ability to produce inhibitory metabolites. For instance, they found that chitinase and β-1, 3-glucanase production by *T. harzianum* which affected the growth of pathogen *S. rolfsii* were influenced by carbon sources (El-Katatny et al., 2000). When *T. harzianum* was grown on laminarin and chitin or on cell walls of the pathogen *S. rolfsii* as sources of carbon, it

excreted β-1, 3-glucanse and chitinase into the medium (Elad and Henis, 1982). Adding nitrogen sources such as peptone and tryptone in the fermentation medium led to increase in chitinase production, whereas adding carbon sources in the fermentation medium noted no additional improvement in chitinase production (Spadaro and Gullino, 2005). Also, Cherif and Benhamou (1990) tested the ability of *Trichoderma* isolated from peat to produce chitinase and inhibit growth of the pathogenic fungus Fol by extracellular metabolites and not by penetration by *Trichoderma* of the pathogen. So, cell walls of Fol were added to liquid cultures of *Trichoderma* and chitinases separated by electrophoresis. At least three major and five minor chitinase activities were produced by *T. harzianum* when it was grown in the existence of cell walls for *F. oxysporum* and these chitinases could be involved in breakdown of the cell wall of Fol.

Also, when *Trichoderma viride* and pathogen *Macrophomina phaseolina* were grown in the dual culture there was a clear zone of inhibition because of antibiosis from both fungi (Baharvand et al., 2014). A previous study indicated that zinc enhanced biocontrol activity by decreasing fusaric acid production by the pathogen FORL consequently, increasing antibiotic production by the biocontrol agent *P. fluorescens* (Duffy and Défago, 1997). A variety of metabolites can be able to inhibit other microorganisms. Phloroglucinols and phenazines have had the ability to inhibit a wide range of fungal pathogens in the laboratory, as well as metabolites can have a specific effect on the particular pathogen, *Agrobacterium radiobacter* produced agrocin 84 which is specific harmful for strains of *Agrobacterium tumefaciens* (Dowling and O'Gara, 1994). The type and levels of antibiotics production usually depended on the carbon sources (Dowling and O'Gara, 1994). Many of variety of metabolites production by fluorescent pseudomonads have ability to inhibit other microorganisms and some of which are engaged in the biological control of plant pathogens (Leisinger and Margraff, 1979). Also, there were 8 known and 3 unknown antibiotics production produced by 16 species of *Aspergillus*, *Pencicillum* and *Fusarium*, by thin layer chromatography(Sawane and Sawane, 2014).

The mycelium growth of *Fusarium oxysporum* f sp. *cucumerinum* was inhibited by antifungal activity of *T. harzianum* 6-penty1-α-pyrone (6PAP). There was 74% and 97% inhibition of FOC when the concentration of the 6PAP was 350 mg/L and 450 mg /L, respectively. Also, 6PAP inhibited the conidia germination of FOC. The percentage of inhibition was 52% at 250 mg /L 6PAP and 96% at 450 mg/L 6PAP. In addition, when 6PAP was added to the

medium with FOC, the population of conidia per ml medium decreased. Moreover, the production of fusaric acid and mycelium growth of FOC was decreased and inhibited by adding diverse concentrations of 6PAP. When 6PAP added to the soil at a concentration of 350 and 450 mg/kg soil, the population of FOC was significantly reduced by 41% and 83%, respectively. Also, the disease indexes of Fusarium wilt reduced from 8 to 15% and from 0 to 5% when addition of 6PAP to the soil at a concentration of 350 mg/kg soil and 450 mg/kg soil, respectively (Chen et al., 2012). The production of volatile and non-volatile compounds by isolates of *Trichoderma* species inhibited the pathogen of Fusarium wilt of chickpea (*Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo and K. Sato) in dual culture (Dubey et al., 2007).

2.2.5. Induced resistance

Induced resistance means stimulating the plant to produce compounds or form barriers locally or systemically that can protect the plant against pathogen, by using chemicals or organisms that are applied to the plants. *T. harzianum* played an important role to induce systemic defense mechanism and mycoparasitism for tomato plants against Fusarium wilt caused by Fol (Mwangi et al., 2011). When *Trichoderma spp*. were applied to the plants one week before inoculation with the pathogenic FORL, plants seemed healthy and without any wilting symptoms (Hibar et al., 2007). Also by using split-root technique to separate pathogen from the antagonist in the soil and on the roots, the half part of root of tomato plant treated with *Trichoderma spp*. had less disease compared to the half part inoculated only with FORL (Hibar et al., 2007). When tomato plants were treated with the *Trichoderma spp*., the colonization of the pathogen in the root tissues decreased. Also, little pathogen hyphae that may penetrate the root system were mostly limited to the epidermis and the outer cortex, while inner tissues were rarely colonized by pathogen (Hibar et al., 2007). Tomato plants treated by *T. harzianum* by adding to soil or dipping of seedling roots, and with salicylic acid and fungicide either separately or in combination, led to protection of tomato seedlings against *F. oxysporum*. In addition, when treated tomato plants before one week from infection by pathogen, the percentage of disease incidence was highly significantly decreased to 0% compared with infection control which was 69% , These results consequently showed induced resistance in tomato plant against pathogen (Houssien et al., 2010).

In split root systems of cucumber plant, plant growth-promoting rhizobacteria (PGPR) strains 89B-27 (*Pseudomonas putida*) and 90-166 (*Serratia marcescens*) induced systemic resistance

against Fusarium wilt (Liu et al., 1995). The induced resistance of tomato plants by non-pathogen *Fusarium* strains against pathogen Fol was related to improved activity of glycosidases and phenol oxidizing enzymes, and increased phenols content (Tamietti et al., 1993). The *Pseudomonas sp*. strain WCS417r induced resistance of carnation plant against Fusarium wilt caused by *F.o. dianthi* and this induced resistance was relative to increased accumulation of phytoalexins in stems (Van Peer et al., 1991). Chickpea plants were resistant to the pathogen *Sclerotium rolfsii*, when plants were treated with *Pseudomonas* strains that induced of phenolic compounds of chickpea plants. *Pseudomonas* strains induced systemic resistance of plants by producing salicylic acid (SA) (Singh et al., 2003).

Also, they found that Fo47 was not only protecting tomato plants from Fusarium wilt, it protected tomato plants from different pathogens such as *Phytophthora infestans* and *Cladosporium fulvum* which means induction by non-pathogens can be nonspecific (Kuc, 1982). Three genes in pepper plants: sesquiterpene cyclase (*CASC1*), a basic PR-1 protein (*CABPR1*) and chitinase (*CACH12*) increased their expression both in the roots and stems during 48 h from treatment by Fo47 with inoculum of *V. dahliae*. Also, two genes *CASC1* and *CACH12* were induced in the stem when plants were treated by Fo47 with inoculation by *V. dahliae*, while just using Fo47 alone for treatment; these two genes were not up- regulated in the stem. So, those genes possibly played an important role in defence of the plant against pathogen by inhibiting growth of the pathogen because those genes increased when the pathogen was present only (Veloso and Diaz, 2012). The suppression of Fusarium wilt by *Pseudomonas fluorescens* WCS417 and *F. oxysporum* Fo47 seemed to be by induced systemic resistance of tomato plants (Duijff et al., 1998).

Nonpathogenic *F. oxysporum* Fo47 played an important role to induce resistance in tomato plants against Fusarium wilt. The disease index for tomato plants infected by *F. oxysporum* was 86%, whereas the disease index was 23% when tomato plants were inoculated by Fo47. This is because Fo47 induced the tomato plant to improve activity of β-1,3-glucanase (+220%), β-1,3-glycosidase (+68%), and chitinase (+26%) in the stem compared with controls (Fuchs et al., 1997). By using a split-root technique to separate pathogen from the antagonist in the soil and on the roots, tomato and watermelon plants were induced to resistance at a partial level by treatment with Fo47 against pathogenic *F. oxysporum* (Larkin and Fravel, 1999). Fo47 assisted flax plants to resist the pathogenic Fo1n3 by collection of electron- dense material in any part infected by Fo47, such as epidermis, hypodermis and

outer cortex. Also, when Fo47 infected the flax plants, it stimulated the cells of plants to produce wall appositions, and cells became surrounded by a layer of cytoplasm with many mitochondria against the hyphal growth of Fo47 (Olivain et al., 2003).

A previous study indicated that non-pathogenic *F. oxysporum* is able to induce resistance against Fusarium wilt in cucumber (Mandeel and Baker, 1991). Non-pathogen *F. oxysporum* induced resistance to Fusarium wilt in chickpea (Hervás et al., 1995). In split root systems, *Fusarium. oxysporum* f. sp. *dianthi* decreased disease symptoms of tomato plant caused by Fol without any direct interaction with this pathogen (Kroon et al., 1991). The growth of Fol was inhibited by combination of elicitors and non-pathogen *F. oxysporum* strain Avr5 and this synergy could contribute to improved fungal resistance in tomato (Amini, 2009). The higher level of induced resistance of watermelon was achieved by a virulent race of *F. o. niveum* compared with *F. o. cucumerinum* (Biles and Martyn, 1989). When the hypocotyl of cucumber plant was inoculated firstly with non-pathogen strain *F. o. cucumerinum*, cucumber leaves were protected from the pathogen *Colletotrichum langenarium* (Ishiba et al., 1981). A previous study showed that the production of antifungal compound in tomato plants was induced due to treating plants with *Fusarium equiseti* (Horinouchi et al., 2011).

2.2.6. Effect of beneficial microorganisms

Arbuscular mycorrhizal fungi (AMF) play an important role to increase nutrient content compared with plants treated with the pathogen, which leads to a decrease in disease. So the existence of AMF in the soil led to a decrease in the disease incidence by pathogen *Fusarium* by about 20%, also AMF spores were more established in the rhizosphere of plant when combined with *Trichoderma viride* (Tanwar et al., 2013). Tomato seedlings transplanted into plastic pots with sterilized soil and infested with pathogen, were treated with *T. harzianum* P52 or AMF singly and in combination (Mwangi et al., 2011). AMF enhanced height and root dry weight significantly when it was applied alone compared to the control. Also, *T. harzianum* had the ability to increase the solubility of phosphate and micronutrients and AMF improved phosphorus and micronutrient uptake in the host plant which led to increased plant resistance to infection (Mwangi et al., 2011). Moreover AMF has an important role in changing the plant rhizosphere environment by shortening the distance between the nutrient and the roots of plant. It means increased ability of plants for absorption (Smith et al., 1986).

The dry root weights and height for tomato plants were improved when *T. harzianum* P52 was applied compared to the control (Mwangi et al., 2011) . Because, *T. harzianum* has the ability to solubilize insoluble or sparingly soluble minerals (Altomare et al., 1999). Growth of cereal crops was improved by *Penicillium spp*. (Whitelaw et al., 1997). Growth of tomato plants was improved by arbuscular mycorrhizal fungi (AMF) in soils with low fertility generally due to increased phosphorous absorption (Johnson et al., 1982; Smith et al., 1986).

Growth of tomato plants was improved significantly by the saprophytic plant growth-promoting fungus (PGPF) *Fusarium equiseti* isolated from turfgrass rhizospheres, which also had ability to suppress many soil-borne diseases, such as *Pythium spp*., *Rhizoctonia solani*, *Sclerotium rolfsii*, *Gaeumannomyces graminis* var. *tritici* and *Cochliobolus sativus* (Hyakumachi, 1994). The population of FORL decreased when tomato plants were treated with *F. equiseti* (Horinouchi et al., 2008; Horinouchi et al., 2007). The disease severity of FORL was decreased from 3 to 2.5 in the presence of biocontrol agent *Pseudomonas fluorescence* CHA0 as well as, the disease severity was reduced when CHA0 was combined with any mineral compared with the mineral alone (Duffy and Défago, 1997).

2.3. Making biocontrol work

2.3.1. Difference between field soil and sterilized soil

There are differences between sterilized soil and soil in the field. When testing the ability of the non-pathogenic strains against pathogens in sterilized soil the result will come from just interactions between them. The field soil has many organisms such as bacteria, fungi, mycorrhiza and others, which can affect the pathogenic and non-pathogenic fungi and that could lead to different results compared with sterilized soil. Edel-Hermann et al. (2009) claimed that after 15 days incubation in disinfected soil, the population density of Fo47 was affected by temperature, whereas in nondisinfected soil there was no effect of temperature on the population density of Fo47. In addition, Fo47 was established at similar high population densities after one year from its introduction at two concentrations, but it survived at lower densities in the nondisinfected soils, correlated to the first concentration at which it was introduced.

2.3.2. Application methods

Different methods of inoculation by non-pathogens result in very different levels of protection. In pot trials, coating the seeds of tomato plants by spores of wild type of *T. harzianum* in non- saline and saline soils led to decrease in wilt disease incidence caused by *F. oxysporum*. In 2000/2001 and 2001 /2002 seasons in field trials, the incidence of wilt disease caused by *F. oxysporum* was reduced to 12 and 8 % respectively when tomato plants were treated with *T. harzianum*, The incidence of untreated was 18 and 19 % respectively (Mohamed and Haggag, 2005). However, in another trial there was no reduction in the disease caused by *F. oxysporum* when *T. harzianum* was applied as a seed coating, whereas soil treatment led to decrease disease incidence by 92% (Ramezani, 2010). Also, the Fusarium wilt of pigeonpea decreased up to 84% when *Trichoderma* was applied by biomass formulation into soil, but disease incidence was only reduced 68.7% by seed treatment (Mohamed and Haggag, 2005). When tomato seeds were treated by *T. harzianum* spores, the disease caused by *Fusarium oxysporum* f. sp. *lycopersici* did not decrease. However, the disease was decreased by 92% when soil was treated by *T. harzianum* (Ramezani, 2010).

Applying non-pathogens as conidial suspension to soil should be better than coating the seeds with non-pathogens as biological control. The reason is that the possibility of non-pathogens colonizing plant roots is bigger than seeds because of the different size between them.

Non-pathogen strain *F. oxysporum* Fo47 reduced the symptoms of Verticillium wilt and Phytophthora blight in pepper plants, when Fo47 was applied to soil as a conidial suspension (Veloso and Diaz, 2012). The inoculum density of pathogen *F. oxysporum* f. sp. *cucumerinum* was reduced when non-pathogen *Fusarium oxysporum* (C5 and C14) was applied to soil at 1.08 x10^4 and 5 × 10^4 colony-forming units (cfu) per gram of soil (Mandeel and Baker, 1991). Adding non-pathogen *F. oxysporum* F47 to soil led to control of FOLR which causes tomato foot and root rot (Bolwerk et al., 2005). Also, Mwangi et al. (2011) showed that applying non-pathogen P52, AMF, AMF+P52 to soil significantly enhanced height and dry weights of tomato plants and reducing Fusarium wilt disease severity compared with untreated plant.

2.3.3. Timing of application

It is very important to focus on the choice of best time of application that can be useful as antagonist and that time depends on things such as kind of pathogen, kind of antagonist, and kind of plant. So previous studies concentrated on time of application of non-pathogens. When tomato plants were inoculated with *T. harzianum* (seedling root dipping and/ or soil treatment) and after one-week plants were inoculated with pathogen *Fusarium oxysporum* f. sp. *lycopersici*, there were completely protected from Fusarium wilt in all applied treatments (Houssien et al., 2010). After 2 weeks from treating tomato seeds with non-pathogen *Fusarium oxysporum* (CS-20 and CS-24), tomato seedlings were transplanted into field soil infested by *Fusarium oxysporum* f. sp. *lycopersici*, the disease was significantly reduced at different range of temperature (Larkin and Fravel, 2002). When non-pathogen *F. oxysporum* was applied to banana plants before field planting, the protection of Cavendish banana was significantly increased against nematode infection (Pocasangre et al., 2000).

The disease severity of *F. oxysporum* (Fo5a4) of tomato plant was reduced when a non-pathogen strain of *F. oxysporum* was applied to roots and soil before inoculating tomato plants with the pathogen (Olivain et al., 1995; Tamietti and Alabouvette, 1986; Tamietti and Pramotton, 1990). Fo47 reduced the extend of Fusarium wilt Fo18 of tomato plants from 54% to 37% when plant roots were inoculated with Fo47 10 days before inoculation with pathogen Fo18 (Fuchs et al., 1997). Fo47 protected pepper plants from infection with pathogen *Verticillium dahliae* when the plant was inoculated with Fo47 before the pathogen (Veloso and Diaz, 2012). Moreover, 48 h after inoculation of the leaves of pepper plants by Fo47 the plants were protected from *P. capsici* by induced systemic resistance (Veloso and Diaz, 2012)

The germinated conidia of Fo47 and Fo18 after 18 h from inoculation were noted in the soil explored by root hairs then the germ tube arrived the same locations on the root surface 2 days after inoculation. After that Fo47 was found alone on younger portions of the root and the root surface became more intensively colonized by hyphae of Fo47 than by hyphae of Fo18 after 3 days from inoculation (Olivain et al., 2006). So, generally non-pathogens should be applied many days before inoculation by pathogen. The reason is to give the non-pathogen time to grow because most non-pathogens can only be active against pathogens after several hours.

2.3.4. Combination of non-pathogenic fungi with organic amendment

After soil incubation for 0-28 months, *T. harzianum* combined with organic amendment at concentration of 6 and 10%, led to reduce Fusarium wilt disease significantly by 21-36% and 29-34% respectively, compared to the control at the corresponding assessment date. Also, fresh weights for tomato plants were increased 14- 44%, when *T. harzianum* was combined with 10% organic amendment compared with using organic amendment alone after 18-28 months of soil incubation respectively. Further, the population of *F. oxysporum* after 18 and 28 months of soil incubation decreased when *T. harzianum* was combined with organic amendment at a concentration of 6 and 10% compared to the corresponding control (Barakat and Masri, 2009). All *Trichoderma* isolates significantly decreased the wilt disease caused by Fol, characterized by leaf chlorosis, wilting, browning of the vascular tissue and ultimately drying up of the leaf. However, *T. viride* combined with arbuscular mycorrhizal fungi (AMF) were more effective to delay the wilt disease than using separately (Tanwar et al., 2013). No one has tested organic amendment in combination with non-pathogenic *Fusarium oxysprum*

2.4. Root exudates

Root exudate is defined as multiple components such as sugar, organic acids, amino acids, peptides, enzymes, vitamins, nucleotides, fungal stimulators, inhibitors and attractants, nematode hatching and attracting factors, and many miscellaneous compounds which are released into the surrounding medium by plant roots. These components have effects on the growth and antifungal activity in the rhizosphere. The quality and quantity of these components released by the root into the rhizosphere are dependent on environmental factors, disease injuries, microbial activities and type of plant. When plant tissue gets damage or roots are attacked by pathogens, toxic aglycones are produced (Friebe et al., 1998). Root exudates of plants contain mostly complex mixtures of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions (e.g. HCO, OH, H), gaseous molecules (COH), enzymes and root border cells (Dakora and Phillips, 2002).

Previous studies showed that the major components of tomato root exudate are sugar and organic acids (Kravchenko et al., 2003) as well as amino acids (Tahat et al., 2010). The major sugars in root exudate of tomato plant are glucose, fructose, and xylose, while the major organic acids in tomato root exudate are citric acid, malic acid and succinic acid (Kamilova et

al., 2006). However, the amount of organic acid was higher than sugar in root exudate of plants, and both of them was increased through plant growth (Kamilova et al., 2006)

Root exudates play a key role in the microbial community. A previous study showed that exotic plant species affected the structure and function of the soil microbial community (Kourtev et al., 2002). Root exudates impacted the soil microbial community in their immediate locality (Bertin et al., 2003). The resistance of cucumber cultivars to Fusarium wilt had a significant effect on the soil microbial community composition and activity and also affected differences in the rhizosphere microbial community (Yao and Wu, 2010). Root exudates have the ability to help as signals for rhizosphere microbes which can influence their behaviors and gene expression as well as root exudate can be helpful as a nutrient (Mark et al., 2005). The populations of rhizosphere microorganisms were significantly higher than the populations of non-rhizosphere and root endophytic microorganisms and this could be due to the exudate supplying an appropriate environment for colonization of the rhizosphere (Naher et al., 2009).

The variety of sugar, organic acids, and secondary metabolites which are secreted from plants can act as chemotactic compounds for plant-associated bacteria (Brencic and Winans, 2005). The microbial activity in the rhizospere was stimulated by organic compounds which were released by the plant roots (Bacilio-Jim´enez et al., 2003). The organic acids secreted from tobacco cultivars had an effect on the colonization of tobacco rhizosphere by pathogenic bacterial strains (Wu et al., 2015) L-malic acid (MA) secreted from roots of Arabidopsis (*Arabidopsis thaliana*) can improve *Bacillus* biofilm formation on the root surface (Rudrappa et al., 2008). Also, citric acid secreted from cucumber plants can stimulate Colonization of *Bacillus amyloliquefaciens* SQR9 on the root surface and motivate its biofilm formation (Zhang et al., 2014). *Pseudomonas chloroaphis* SPB1217 and *P. fluorescens* SPB2137 grew on sugar more poorly than on organic acids, whereas when organic acids and sugars were mixed together, the growth of rhizobacteria was good (Kravchenko et al., 2003). Although *P. fluorescens* WSC365 and *P. fluorescens* OE28.3 were good users of sugars, the colonization on the tomato root was poor for both of them, therefore there was another major carbon source for colonization such as organic acids and polymer (Lugtenberg et al., 1999).

Previous studies showed that root exudate has an effect on spore germination. Spore germination of *Gigaspora albida* was suitable in the water-agar medium at 7 days, followed by the c- salt medium of Murashige & Skoog (MS) at 14 days. However spore germination

was not high in the sand and root exudate of *Panicum miliaceum* medium (Maia and Yano-Melo, 2001). Root exudate of strawberry plants decreased sporulation of the oomycete *Phytophthora fragriae* in vitro studies (Norman and Hooker, 2000). Root exudate of non-mycorrhizal tomato plants improved spore germination of AMF compared to water only, however, the spore germination was more than double in the presence of root exudate from mycorrhizal plants compared to spore germination in the presence of root exudates from non-mycorrhizal tomato plants (Scheffknecht et al., 2006). Root exudates of *Asparagus densiflorus* released peroxidase and phenylalanine ammonialyase and these enzymes may inhibit fungal spore germination and germ tube elongation of *Fusarium oxysporum* f. sp. *asparagi* (He et al., 2001). The root exudate of *Pinus resinosa* inhibited 80% of the sporulation of *F. oxysporum* when plants were treated with ectomycorrhizal fungus *Paxillus involutus* (Duchesne et al., 1988). The stimulation of microconidia germination of FORL and Fol was similar in the presence of tomato root exudates, whereas the degree of spore germination response differed in separate *F. oxysporum* strains (Steinkellner et al., 2005). Root exudates of tomato plants played an important role to increase microconidial germination rate of biocontrol strain Fo47 challenged with the tomato wilt pathogen Fol007 (Steinkellner et al., 2008). Also, they found that the percentage of spores of biocontrol strain Fo47 was higher than the pathogen FORL in present of tomato root exudate (Bolwerk et al., 2005)

Root exudate could play an important role in stimulating plant pathogens which leads to disease (Whipps, 2001a). However, plant root exudates can supply carbon compounds to stimulate specific microbial populations in rhizosphere and improve bio-control efficiency (Morgan et al., 2005). The ability of rhizosphere fungi to inhibit growth of the pathogen *F. oxysporum* f.sp. *pisi* increased ten times in presence of root exudate of pea (Buxton, 1960). The growth of pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt) was inhibited by combined root exudate of *Triticosecale spp*. and rye (*Secale cereale*) with nutrient media, and also they found that the inhibitory effects of similar extracts from two wheat cultivars were different (Wilkes et al., 1999) Also, the 6-methoxybenzoxazolin-2(3H)-one (MBOA) secreted from wheat had the ability to reduce the mycelial growth of *Gaeumannomyces. graminis* var. *tritici*, *Cephalosporium. gramineum*, and *Fusarium. culmorum* by 50% (EC50) at the concentrations of 77, 134, and 271 µg/mL of corn meal agar, respectively (Martyniuk et al., 2006). Agar medium containing root exudate of Otto, Domo, Dollinco and Tukán cultivars of

wheat without nutrient inhibited growth of *Gaeumannomyces graminis* var. *tritici* (Schalchli et al., 2012).

2.5. Conclusion

Biological control is one of the most important ways to control plant diseases without harming the environment or human safety. Non-pathogenic strains of *Fusarium oxysporum* have been shown by many workers to decrease the growth of pathogenic *F. oxysporum* and reduce the incidence of Fusarium wilt and root rot diseases. However other researchers found that non-pathogens did not have any effect on the pathogen. So more work is needed to understand the factors that affect biological control so that it can be made more successful. Different investigations have reported effects of factors including temperature, light and soil, nutrients and host root exudate on growth of fungi and on the antagonism between non-pathogen and pathogen. An increase or decrease in some of these factors improved the antagonism of non-pathogen against pathogen. However, some factors had a negative effect on the non-pathogen. Also, workers have tested the ability of non-pathogens to induce resistance of plants against the disease by diverse ways. The researchers also found that environmental and host factors had effects on induced resistance by non-pathogens. Most previous studies focused on the percentage of plants affected by the pathogen and they did not study effects on the growth of plants. Despite the large body of literature on biological control of diseases caused by *F. oxysporum*, there is a need for more work to fully understand and improve this system.

Chapter 3. Isolation and screening of non-pathogenic *Fusarium oxysporum*

3.1. Introduction

The review of the literature showed that non-pathogenic strains of *F. oxysporum* had great potential as biocontrol agents for Fusarium wilts (Larkin and Fravel, 2002; Tamietti and Alabouvette, 1986; Tamietti and Pramotton, 1990). In order to do experiments that might improve the effectiveness of this method, it is necessary to obtain suitable non-pathogen isolates. Although strains like Fo47 have shown an effect against many different Fusarium wilt diseases, it is likely that non-pathogens isolated from soil or rhizosphere of tomato will be well adapted to tomato cropping systems.

Traditionally, fungal biocontrol agents have first been screened in dual culture assays. However, as pointed out by Fravel et al. (2003), the best and most reliable method for screening antagonists of Fusarium wilts is in bioassays with the plant and pathogen. These require a reliable method of infecting the plants. Pathogenicity tests for vascular wilts are commonly done using the root dipping technique, where seedlings are uprooted and the root systems soaked in a spore suspension before transplanting into soil. Less disruptive alternatives include adding spore suspensions directly to the soil. The best method for setting up bioassays needs to be determined.

Disease rating for vascular wilts is commonly done by measuring incidence or severity of wilting symptoms (Houssien et al., 2010; Larkin and Fravel, 1998). However, incidence requires the use of a large number of plants, and wilting is a late-stage symptom that requires environmental stress that is difficult to achieve in a glasshouse. An alternative method is to measure the extent of browning of the vascular system. Infection with vascular wilts will also reduce the rate of plant growth by reducing transpiration and nutrient transport, and this could be a suitable method for assessing the effect of pathogens and antagonism by non-pathogens.

Non-pathogenic strains of *F. oxysporum* are generally assumed to have no adverse effects on the plant growth because they do not cause wilting (Bao et al., 2002; da Silva and Bettiol, 2005). However, it is possible that they do have negative effects on sensitive stages, such as germination and seedling growth. The effect of antagonists in the absence of the pathogen needs to be examined.

The aim of the experiments described in this chapter was to isolate and characterize strains of *F. oxysporum* that were suitable for more detailed experiments on antagonism of Fusarium wilt of tomato. Fungi were isolated from tomato soil, identified using morphological and molecular methods, screened for antagonistic ability in bioassays, and tested for their effect on seed germination and growth.

3.2. Material and Methods

3.2.1. Isolation and identification of isolates

Fungi were isolated from the roots of tomato plants and surrounding soil collected from a clay soil in Armidale, NSW, Australia. All plants were free of Fusarium wilt symptoms. The roots were washed in tap water to remove all visible soil and then transported in plastic bags to the laboratory.

At the laboratory, the roots were cleaned of any remaining soil by first washing them with sterile water. The roots were then surface sterilized with 1% sodium hypochlorite for 1 min and 75% ethanol for 30 sec then washed three times with sterilized water. Other roots were washed with sterilized water for isolation of rhizoplane fungi and air dried on sterilized filter paper. The roots were cut into pieces that were 1 cm long using a sterile blade. The pieces were then placed on Komada medium (Belgrove et al., 2011). The Petri dishes were incubated at 25°C and checked daily for fungal growth for 7 days. Tomato field soil was diluted with distilled water and 1 ml from a 10^{-4} dilution was poured onto Petri dishes with Komada medium. Then emerging colonies were transferred onto fresh potato dextrose agar (PDA). *Fusarium* isolates were subcultured for 7 days on synthetic nutrient agar (SNA). This is a medium that allows for sporulation of *Fusarium* spp. (Mwangi et al., 2011). *F. oxysporum* was identified to species level based on morphology of microconidia, macroconidia, conidiophores, and chlamydospores. Typical isolates of *F. oxysporum* were kept for testing their biocontrol potential as described below. Isolates were then preserved in slant culture on PDA under sterilized water at 4°C (Subhani et al., 2013).

3.2.2. Pathogens

Cultures F3445, F5766, F5768 and F5769 of Fol were provided from the culture collection of the Royal Botanic Gardens, Sydney, by Dr Matthew Laurence. Stock cultures were prepared

as for the non-pathogen isolates. Pathogenicity of the isolates was confirmed by observation of wilting of tomato seedlings following root-dip inoculation (Bao et al., 2002; Horinouchi et al., 2011).

3.2.3. Molecular identification

Identification of *F. oxysporum* isolates was confirmed by obtaining partial sequences of the translation elongation factor 1α gene. For DNA extractions, isolates were grown on PDA medium for 7 days at 25°C at room temperature. Mycelium was then scraped directly from agar plates and used for DNA isolation. Total genomic DNA was isolated using the ISOLATE II Plant DNA kit (Bioline, Sydney, Australia). Sequences were amplified by PCR using ef1 and ef2 primers with an annealing temperature of 53°C (Geiser et al., 2004) with the Bioline MyTaq mix. PCR products were cleaned up with the Bioline ISOLATE II PCR and Gel kit, and sequenced in both directions using the PCR primers by the Australian Genome Research Facility, Sydney. Representative sequences from the analysis of O'Donnell et al. (1998) were downloaded from GenBank. All sequences were aligned using ClustalW and a maximum likelihood tree was calculated using MEGA 6.0.

3.2.4. Inoculum preparation

Fungal cultures were grown on PDA in 9 cm Petri dishes for 6 days in the dark at 25°C. Six mycelial discs (diameter 5mm) taken from the edges of the cultures were transferred into 250 ml potato dextrose broth in 500 ml flasks and incubated for seven days at 25°C in a rotary shaker at 120 rpm. After 7 days of culture, the mycelial mats were removed by filtration through Miracloth (Millipore). The microconidia were resuspended in sterile distilled water. The density of the conidial suspension was adjusted to 10^6 spore/mL using a haemocytometer (Horinouchi et al., 2011; Olivain and Alabouvette, 1997).

3.2.5. Comparison of inoculation methods for pathogen

Isolates of the pathogen were tested on the tomato cultivar Grosse Lisse which is susceptible to race 1 of Fol. Tomato seeds were sown into plastic trays (10 x 15 x 5(height) cm), containing non sterile soilless potting mix (Searles Premium Potting Mix, Searles, Kilcoy QLD, mixed 1:1 with washed river sand) and were allowed to grow for 3 weeks in a glasshouse at 25°C (day) and 20°C (night) with natural light. The first way of testing pathogenicity used a root dip inoculation method. Tomato seedlings were uprooted gently

and roots were washed with tap water to remove all soil. Roots were dipped in a suspension containing 10^6 spores/mL for 1 h, dried briefly on a paper towel, and then one plant transplanted into plastic pots containing non-sterile soilless potting mix (16 cm diameter x 17 cm height). Control seedlings were dipped with water alone. The second way of testing the pathogenicity was adding conidial suspension to tomato seedlings transplanted in the same pots as the first method. Conidial suspension was added at the rate of 200 ml of 10^6 conidia/ ml to 2 kg of potting mix. There were three replicate pots for each fungal strain. Control seedlings were treated with water alone. Plants were placed in a glasshouse under the same growth condition as described above. Plants were evaluated every 3- 4 days for 3 weeks for symptoms of wilting (Bao et al., 2002) and then harvested to determine height, and root and shoot dry weights.

3.2.6. Antagonism of mycelial growth of pathogen in vitro

The seven isolates of *F. oxysporum* from tomato roots and soil were tested for their efficacy to inhibit growth of isolate F5769 of Fol in dual culture on PDA medium. Both the antagonist and the pathogen were simultaneously inoculated at the opposite ends 5 cm apart from each other in the Petri dishes containing about 20 ml of PDA medium in triplicate. Mycelial plugs of 5 mm diam. from the margins of 6 day old cultures of pathogen and non-pathogen were used. In controls the pathogen was inoculated alone near one edge of the Petri dish. Inoculated Petri dishes were incubated at 25°C for 7 to 10 days. The percent inhibition of fungal growth of the pathogen was calculated using the following formula

$$Inhibition = \frac{R1 - R2}{R1} \times 100$$

Where R1 is the radial growth of the pathogen in the control and R2 is the distance grown by the pathogen on a line between inoculation positions of the pathogen and antagonist (Ramezani, 2010; Subhani et al., 2013; Whipps, 1987).

3.2.7. Effect of antagonists on Fusarium wilt and growth of tomato

Tomato seeds of the cultivar Grosse Lisse were planted in plug trays (plug size 3.4 by 3.4 by 5 cm; 6 plugs/tray), containing non-sterile soilless potting mix (Searles Premium Potting Mix, Searles, Kilcoy QLD, mixed 1:1 with washed river sand). 3 ml conidial suspensions of approximately 10^6 conidia/ml of non-pathogens (F1, F2, F3, F4, F5, F6, F6 and F7) were added to each plug cell and control plug cells were left untreated. After two weeks, plugs containing the tomato plants were transplanted into 16 cm diameter pots containing non sterile soil potting mix infested with the Fusarium wilt pathogen. Pathogen inoculum consisted of isolate F5769 of Fol. Inoculum consisting of conidia was added to each pot at a rate of 200 ml of approximately 10^6 conidia/ml of pathogen. For each treatment there were six replicate pots of 1 plant /pot. Disease was observed after 9 weeks and was assayed as the length of brown colour in the vascular tissue after the stem had been cut lengthwise (Larkin and Fravel, 2002; Mwangi et al., 2011)

In a second experiment, tomato seeds were grown as above. After 2 weeks tomato plants were transplanted into 16 cm diameter pots containing non-sterile soilless potting mix. After one week conidial suspensions (200ml of approximately 10^6 conidia/ml) of non-pathogen F1 and F4 and pathogens F5768, F5766 and F3445 were added to 2 kg of potting mix for each pot. Each treatment consisted of four replicate pots of 1 plant /pot. Disease was observed by comparing growth between treatments and control (pathogen only). After 6 weeks height, shoot dry weight and root dry weight were determined. Shoot height was taken from the base of the stem to apex. The shoots and roots were dried in an oven at 70°C until constant weight (Larkin and Fravel, 2002; Scher and Baker, 1982).

3.2.8. Effect of antagonists on seed germination

Seeds of tomato cv. Gross Lisse were surface sterilized with 1% sodium hypochlorite for 1 min then washed three times with sterilized water and put on sterilized filter paper sheets for drying. Ten tomato seeds were sown in each 16 cm diameter pot containing non-sterile potting mix. Inoculum of F1 and F4 was prepared in the form of a conidial suspension and added to each pot at a rate of 200 ml of approximately 10^6 conidia/ml. Seeds without any inoculation served as control. Plants were watered regularly. After 21 days from sowing,

percent seed germination was determined (Alwathnani and Perveen, 2012; Alwathnani et al., 2012).

To test whether inhibition of germination was due to antibiotic production, F1 and F4 were separately cultured on potato dextrose agar in 9-cm Petri dishes for seven days in the dark at 25°C.Then 5 mycelial disks (diameter 5 mm) taken from the edges of the cultures were transferred into 150 ml of potato dextrose broth in 500-ml flasks and incubated at 25°C in a rotary shaker at 120 rpm for 2 days and 180 rpm for 19 days. The fungal biomass was removed by dual filtration through filter paper, and then culture filtrates of the fungal strains were filter-sterilized by Millipore 0.45 µm syringe filters. Seeds were sterilized as above. Ten tomato seed were placed in each Petri dish. 5 ml of culture filtrate was poured into each Petri dish and 5 ml of sterilize water for control. Seeds were allowed to germinate for 20 days. Each treatment had five replicates.

3.2.9. Statistical analysis

Data were analyzed by ANOVA, using $P < 0.05$ as the criterion for significance. Growth data were log-transformed when necessary to ensure homogeneity of variance. When the assumptions of ANOVA could not be met, treatments were compared with the Kruskal-Wallis test.

3.3. Results

3.3.1. Isolation and identification of isolates

Non-pathogen fungi were isolated from tomato roots and tomato field soil. Overall seven *Fusarium* isolates (F1, F2, F3, F4, F5, F6 and F7) were collected. Based on colony and spore morphology, all 7 isolates were identified as *F. oxysporum*.

Comparison of partial translation elongation factor 1α sequences with those from representative strains of *F. oxysporum* showed that all sequences from the pathogen isolates were identical with that of Fol NRRL 26034 (Figure 3-1). The non-pathogen isolates clustered in two groups. F1, F3, F5 and F6 were in one group similar to sequences from formae speciales *cubense*, *vasinfectum* and *melonis*, while F2, F4 and F7 were in a second group similar to sequences from formae speciales *canariensis*, *cubense* and *perniciosum*

(Figure 3-1). Sequences from the non-pathogen isolates F1-F7 were dissimilar to all sequences from Fol or FORL.

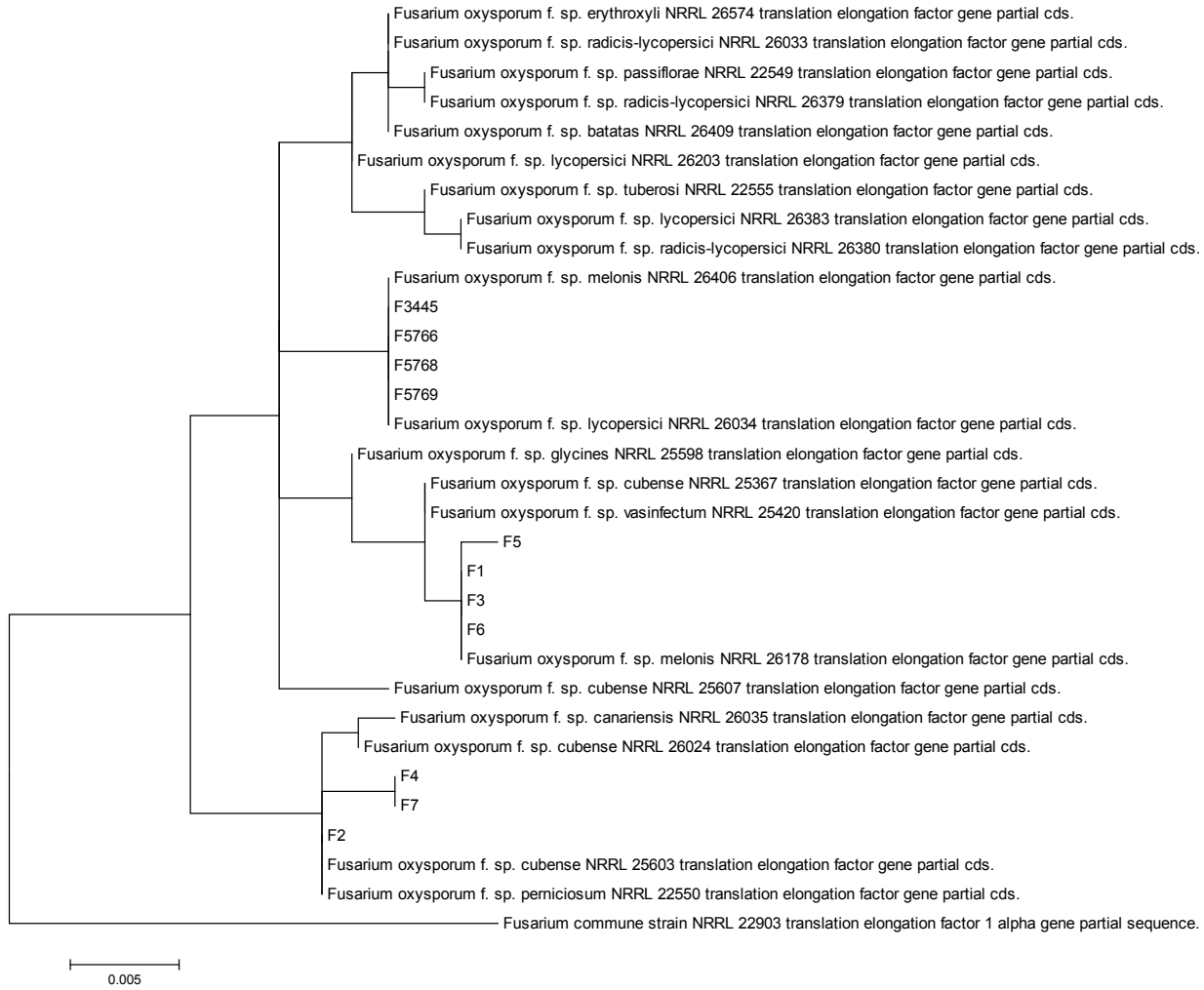


Figure 3-1 Maximum likelihood tree showing relationships between partial translation elongation factor 1α sequences from pathogens (F3445, F5766, F5768, F5769) and non-pathogens (F1-F7) used in this study and representative sequences from the analysis of O'Donnell et al. (1998).

3.3.2. Comparison of inoculation methods for pathogen

There was a significant difference between root dipping and adding conidial suspension of pathogens F3445 and F5768 to tomato plants. Disease severity of pathogens by using root dipping was stronger than adding conidial suspension to the soil (Figures 3-2, 3-3, 3-4). All treatments showed significant decrease in plant height compared to untreated plants. However when pathogens F3445 and F5768 were applied by root dipping, the height of the plant was reduced by more than 70% compared with plants treated by adding conidial suspension of F3445 and F5768 to soil (Figure 3-2). The biggest effect of F3445 and F5768

on the shoot and root dry weight was recorded in root dipping, with the plants showing very low growth (Figures 3-3, 3-4). Adding conidial suspension of F3445 and F5768 to the soil showed lower, but still significant, effects on the shoot and root dry weight. Also, tomato plants showed wilt when F3445 and F5768 were used for root dipping, while adding conidial suspension of F3445 and F5768 did not show wilt on the tomato plants.

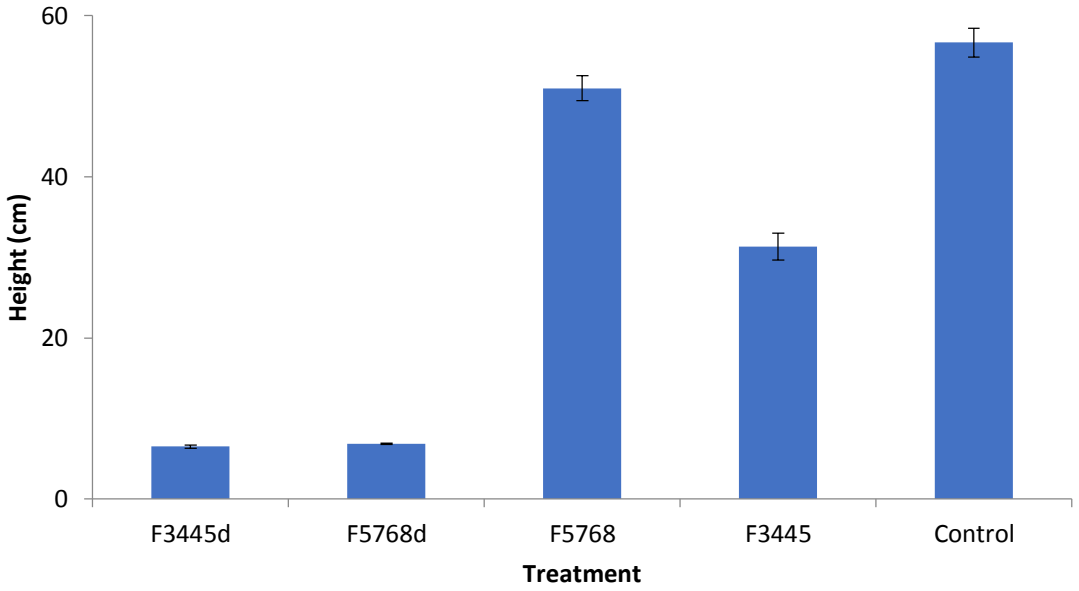


Figure 3-2 Effect of pathogens F3445 and F5768 on the height of tomato plants after inoculation by root dipping (F3445d, F5678d) or adding conidial suspension to soil. Error bars show standard errors (n=3)

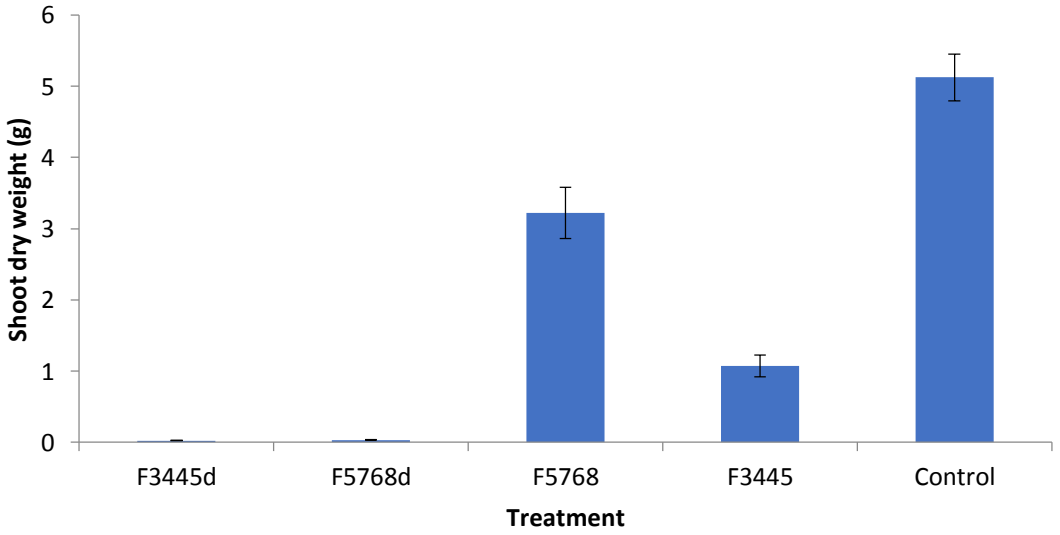


Figure 3-3 Effect of pathogens F3445 and F5768 on the shoot dry weight of tomato plants after inoculation by root dipping (F3445d, F5678d) or adding conidial suspension to soil. Error bars show standard errors (n=3)

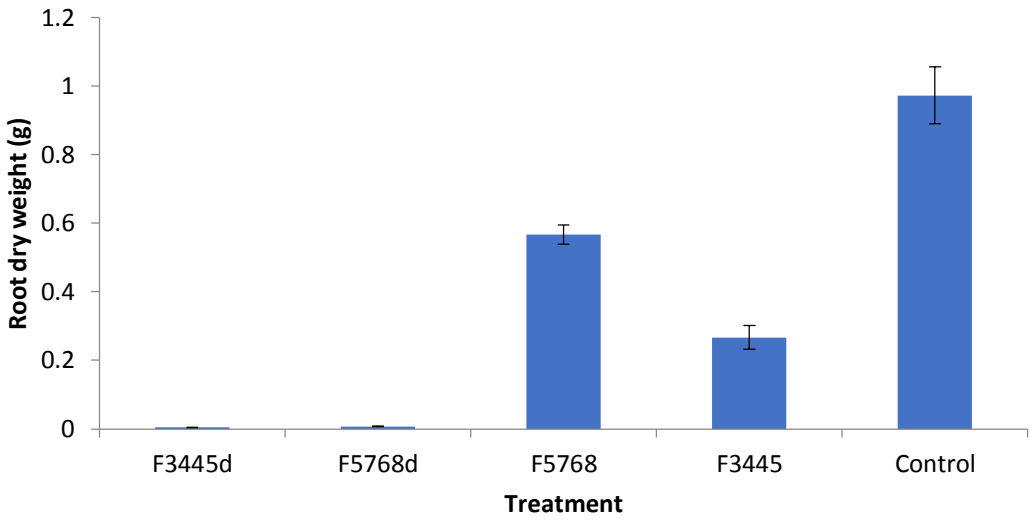


Figure 3-4 Effect of pathogens F3445 and F5768 on the root dry weight of tomato plants after inoculation by root dipping (F3445d, F5678d) or adding conidial suspension to soil. Error bars show standard errors (n=3)

Figure 3-5 Effect of pathogens F3445 and F5768 on the tomato plant by using root dipping

3.3.3. Antagonism of mycelial growth of pathogens in vitro

All tested non-pathogen isolates inhibited the colony growth of pathogen F5769 on PDA to varying degree (Figure 3-6). The highest inhibition of growth of pathogen was recorded in F4 (59%) followed by F3, F6 (55 and 53%) respectively, while the lowest inhibition of growth of pathogen was recorded in F7 (37%) followed by F2 (46%).

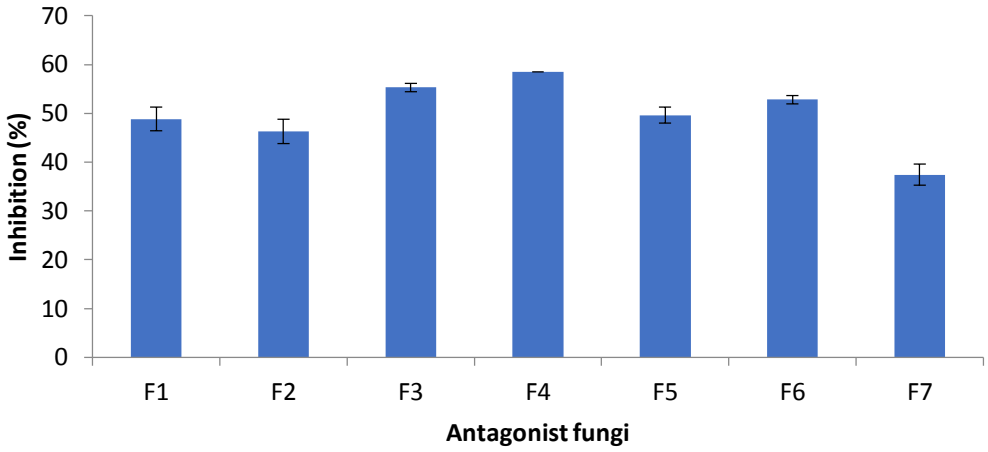


Figure 3-6 Inhibition of growth of *F. oxysporum* f. sp. *lycopersici* F5679 by antagonistic fungi in dual culture. Error bars show standard errors (n=3)

3.3.4. Effect of antagonists on Fusarium wilt and growth of tomato

Tomato seedlings treated with non-pathogenic isolates two weeks before inoculation with the pathogen had reduced disease severity of Fusarium wilt shown through different levels of brown colour in the stem. The amount of browning differed between fungal treatments ($P < 0.001$, Kruskal-Wallis test). F1 and F4 protected all the plants from browning by the pathogen. There was no brown colour for all six replicates for each of F1 and F4. However, the other non-pathogens did not protect plants completely. F3 and F7 protected 4 and 5 plants respectively against pathogen, while the other non-pathogens protected less than half of the plants from browning.

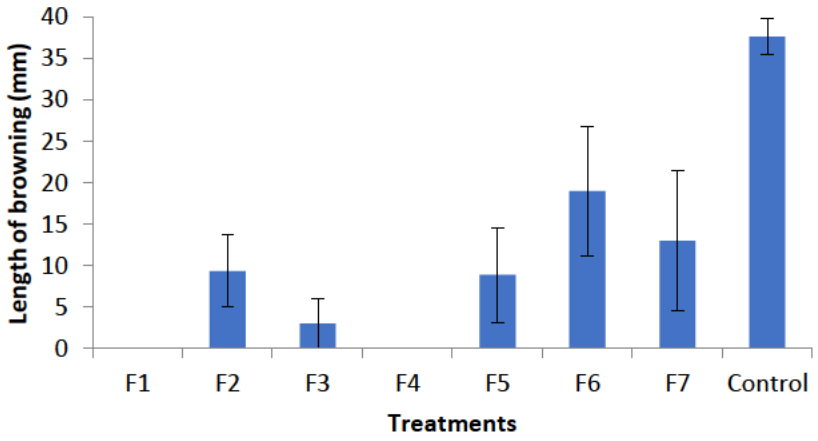


Figure 3-7 Effect of antagonists on disease severity of Fusarium wilt by measuring the length of brown discoloration in the tomato stem caused by pathogen F5769. Error bars show standard errors (n=6)

In pot culture under glasshouse conditions, there was a significant effect of antagonists F1 and F4 on the height, shoot and root dry weight of tomato plants compared with pathogen only. Both F1 and F4 increased height, shoot and root dry weight of plants inoculated with each pathogen. However, F1 and F4 reduced the growth of plants in the absence of the pathogen. Both antagonists had similar effects on the height of plants (Figure 3-8). Also, the non-pathogens enhanced shoot and root dry weight of infected plants (Figures 3-9, 3-10). F1 and F4 were more suppressive to pathogen F5768 than to F3445 and F5766.

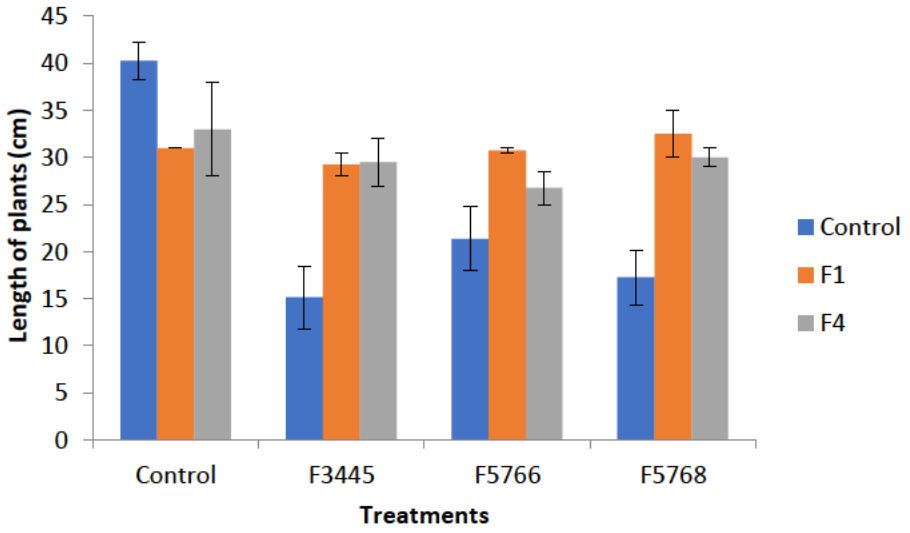


Figure 3-8 Effect of antagonists F1 and F4 on height of tomato plant when introduced into conductive soil infested with pathogen F5768, F5766 and F3445 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=4)

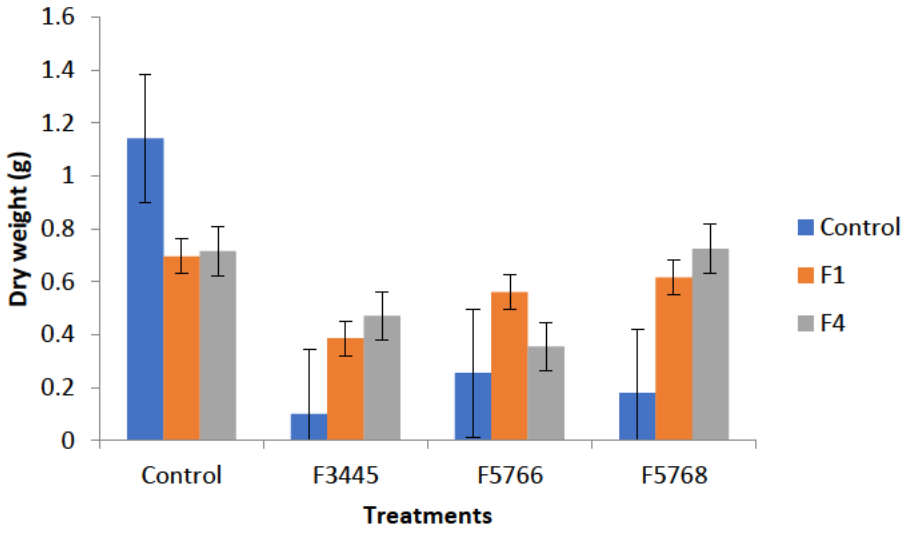


Figure 3.9 Effect of antagonists F1 and F4 on shoot dry weight of tomato plant when introduced into conductive soil infested with pathogen F5768, F5766 and F3445 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=4)

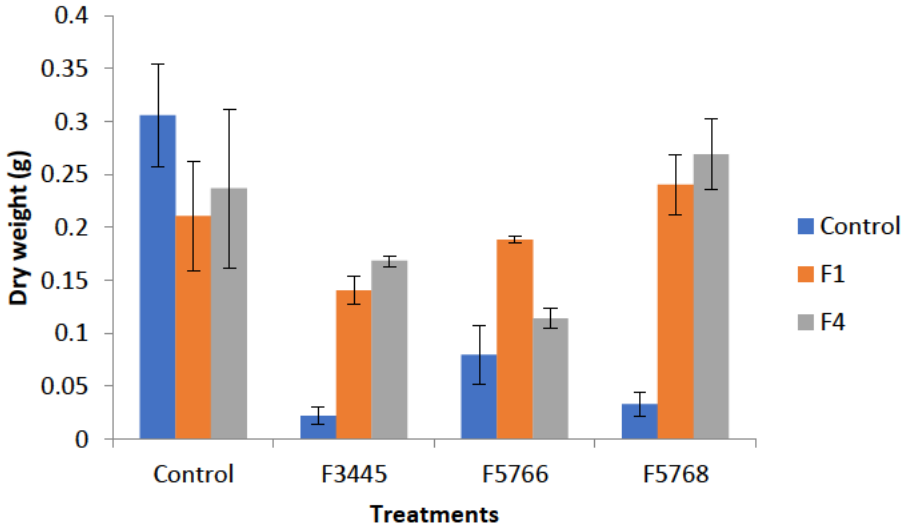


Figure 3-10 . Effect of antagonists F1 and F4 on root dry weight of tomato plant when introduced into conductive soil infested with pathogen F5768, F5766 and F3445 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=4)

3.3.5. Effects of antagonists on seed germination

The percentage of germination in pots of tomato seeds treated with non-pathogen (F1 and F4) was significantly reduced compared with untreated control. F1 reduced germination by 51% and F4 by 44% (Figure 3-11).

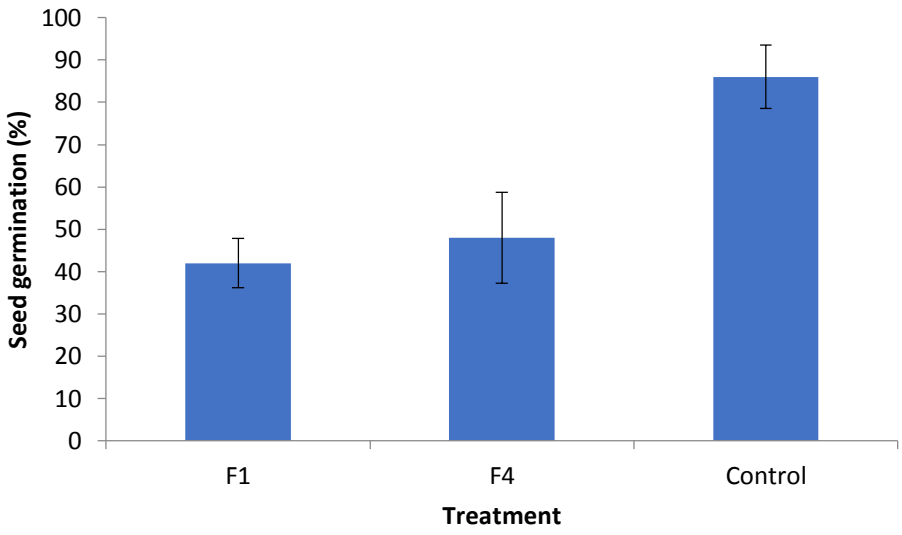


Figure 3-11 Effect of antagonists F1 and F4 on seed germination of tomato when introduced into soil (200ml of approximately 10^6 conidia/ml). Error bars show standard errors.

Toxic metabolites in the culture filtrates of both antagonists F1 and F4 caused reduction in seed germination (Figure 3-12). The inhibition of the germination of tomato seed was 43% for F4 and 34% for F1.

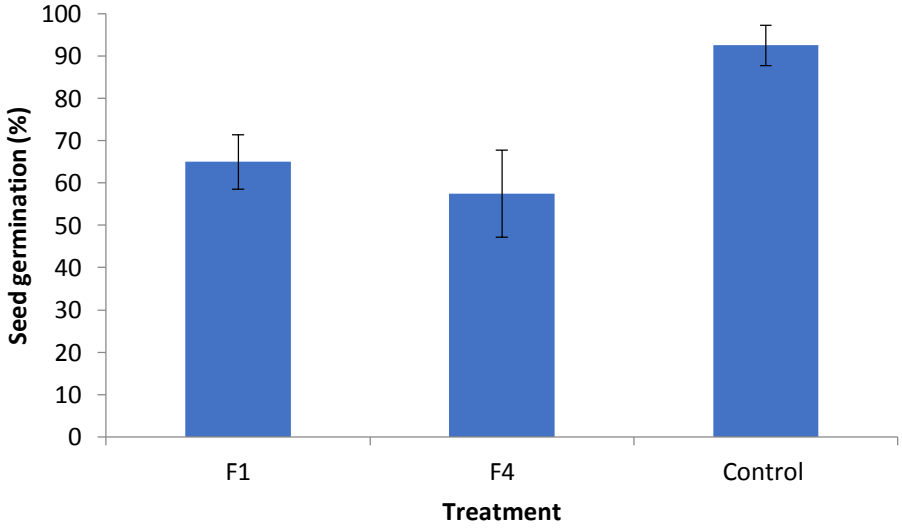


Figure 3-12 Effect of culture filtrate of F1 and F4 on seed germination of tomato in Petri dishes. Error bars show standard errors.

3.4. Discussion

In this study, I examined specific *F. oxysporum* associated with tomato plants, including non-pathogen strains isolated from plant roots. Colony and spore morphology identified the non-pathogens. The sequencing of 340-bp fragment of the tef-1α gene confirmed the identification. One outcome of this investigation was that 7 putative non-pathogens separated into two groups that were not closely related to the pathogen isolates.

Disease severity of pathogens by using root dipping was stronger than adding conidial suspension to the soil. This could be because the conidial suspension of pathogen has direct contact with plant roots in root dipping. So, the spores can penetrate the root directly through the epidermis or root hairs. However, adding conidial suspension of pathogen to soil does not have direct contact of spores with roots, so the fungus reaches the xylem more slowly. When roots were dipped into a conidial suspension of Fol8, the ability of pathogen Fol8 to attach to the root surface and reach to the elongation zone and develop disease was greater than by adding conidial suspension to soil (Olivain et al., 2006). This result agreed with Shaw et al. (2016) who found that when castor (*Ricinus communis*) was inoculated with *Fusarium oxysporum* f.sp. *ricini* by root dipping method, the percentage of dead plants was higher compared with soil drenching methods, and also the days to death of plants in the root dipping method were less compared with soil drenching methods. So conidial suspension is a better method to use because it allows a measurable reduction in growth without being too severe.

There was a significant difference between antagonists in inhibition of the colony growth of pathogen F5769 on PDA. The highest inhibition of growth of pathogen was recorded in F4 and the lowest inhibition growth of pathogen was recorded in F7. This interaction between non-pathogen and pathogen could be because of production of antibiotics or competition for nutrients that have an effect on the growth of the pathogen. This result is supported by Lemanceau et al. (1993) who found that non-pathogen *F. oxysporum* Fo47b10 competed with the pathogen *F. oxysporum* WCS816 for glucose and this reduced growth of the pathogen. The different effects of non-pathogens on the pathogen could be depended on the strain. For example, previous studies by Kumar and Garampalli (2013) found that in dual culture, the antagonistic activity of isolates of *Trichoderma* against Fol was different.

Non-pathogens reduced disease severity of Fusarium wilt through showing different levels of brown colour in the stem. Non-pathogens collected from tomato root and soil samples were not equally effective in reducing disease incidence. F1 and F4 protected all the plants from infection by the pathogen. There was no brown colour for all six replicates; however, other non-pathogenic strains did not protect plants completely. This observation is also in agreement with previous results obtained by Postma and Luttikholt (1996) who showed that some non-pathogenic *F. oxysporum* had ability to reduce disease severity of pathogen strain *Fusarium oxysporum* f. sp. *dianthi* by reducing the stem colonization by the pathogen. In pot culture under glasshouse conditions, there was a significant effect of antagonists F1 and F4 on the height, shoot and root dry weight of tomato plant compared with the pathogen only.

This result could be because F1 and F4 were more suppressive to the pathogen than other non-pathogens by competing for nutrients or producing antibiotics. Non-pathogens may also stimulate tomato plants to induce resistance against pathogens which I tested in later experiments. This result agreed with Fravel et al. (2003) who reported that non-pathogenic *F. oxysporum* had ability to limit pathogens by many modes of action, such as competition for nutrient in the soil, competition for infection sites on the root and inducing systemic resistance.

The results showed that non-pathogen F1 and F4 improved growth of tomato plants infected with Fol, except F4 with pathogen F5766. So non-pathogens may not be equally effective against all pathogen strains. This result is confirmed by Ordentlich et al. (1991) who found that non- pathogen *Trichoderma harzianum* isolates had different effect against pathogens *Fusarium oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *vasinfectum*.

Also, results showed that F1 and F4 reduced the growth of plants in the absence of pathogen. This could be due to F1 and F4 having the ability to colonize root tissue and the fungi may consume some of the carbon sources from tomato roots, so this could cause reduction in growth of plants. This result is confirmed by Bao and Lazarovits (2001) who found that non-pathogenic *Fusarium oxysporum* 70T01 colonized root tissue of tomato plants but only in the outside of cortex cell layers.

The percentage of germination of tomato seeds treated with non-pathogen (F1 and F4) was significantly reduced compared with untreated control. This result could be because non-pathogens produce secondary metabolites which have an effect on the percentage

germination. Owolade et al. (2005) reported that *Fusarium spp*. can produce over 100 secondary metabolites which are dangerous to maize seed consumers. Also, this result agreed with Jasnic et al. (2005) who found that for soybean seed inoculation on filter paper, all of the isolates of fungi from the genus *Fusarium* significantly decreased the average number of healthy seed (10-12.5) compared to the uninfected control treatment (14.25), while *F. oxysporum* isolate S/1 was the most aggressive against seed germination compared to the other *Fusarium spp*. The percentage germination of the seeds treated with culture filtrates of F1 and F4 was lower than control. This result could due to non-pathogens produced toxic metabolites which reduced germination of seeds. Garuba et al. (2014) found that when seeds were treated with culture filtrate of *Aspergillus niger* and *Penicillium chrysogenum*, the percentage germination of seeds for both was lower than control. Also, culture filtrate of *Fusarium solani*, *F. oxysporum*, *Aspergillus flavus*, *A. niger*, *Alternaria terreus*, *A. alternata* and *Ulocladium botrytis* reduced percentage of seeds germination (Ibraheem et al., 1987).

The outcome of these experiments was that 2 unrelated non-pathogens were chosen, F1 and F4 for further experiments. F1 and F4 had the ability to reduce disease severity much more than the other non-pathogens. Conidial suspension was chosen as the best method to use for inoculation. The antagonists need to be applied after germination, for example at transplanting, to avoid problems with seed germination.

Chapter 4. Induced resistance

4.1. Introduction

Induced resistance of plants towards pathogen infection can be developed by treating plants with a variety of abiotic and biotic inducers (Walters et al., 2005). Plant growth promoting rhizobacteria (PGPR) strains showed more efficacy by inducing systemic resistance against several pathogens attacking the same crop (Ramamoorthy et al., 2001). Induction of systemic resistance can lead to the direct activation of defense-related genes. Induced systemic resistance has been related with the stimulation of several potential defense mechanisms including increased activity of chitinases, β-1,3-glucanases, peroxidases, and other pathogenesis-related (PR) proteins; accumulation of antimicrobial compounds such as phytoalexins; and formation of protective biopolymers such as lignin, callose, and hydroxyproline rich-glycoproteins (Sticher et al., 1997). Priming can also play an important cellular mechanism in induced systemic resistance of plants which leads to an increased response to infection by pathogens (Conrath et al., 2001). So, induced systemic resistance is an important component of disease resistance that improves the health of plants.

Duijff et al. (1998) reported that the absence of any direct contact between the pathogen and the biocontrol strains prevents any microbial antagonism. The disease suppression recorded with inoculation of different parts of split plants could then only be ascribed to a systemic induced resistance of the host plant.

Induced resistance to Fusarium wilt in tomato plant by non-pathogenic *F. oxysporum* has been studied. The chitinase, β-1, 3-glucanase, and β-1, 4-glucosidase activity was increased in the presence of non-pathogen Fo47 (Fuchs et al., 1997). Also, in a split root system, three isolates of nonpathogenic *F. oxysporum* induced systemic resistance in tomato and watermelon plants, but were diverse in relation to their abilities to reduce disease (Larkin and Fravel, 1999). In a split root system in tomato, each half was separately inoculated. So, there was no direct interaction between pathogen and non-pathogen. One half was first inoculated with *F. oxysporum* f. sp.*dianthi* (non-pathogen of tomato) or treated with water, then the second half was inoculated after a week with Fol or by a water treatment. The disease symptoms in the half firstly inoculated with *F. oxysporum* f. sp.*dianthi* were significantly

delayed, compared to plants of which that half had been treated with water (Kroon et al., 1991).

Applying strain WCS374 of *P. fluorescens* to radish plants gave highest induced systemic resistance toward disease (Fusarium wilt) when iron availability in the radish nutrient solution was low, compared with high iron (Leeman et al., 1996). So this study was focused on finding whether non-pathogens can induce resistance of tomato plant against pathogen, as well as studying the role of some nutrients such as Fe on the induced resistance.

4.2. Materials and methods

4.2.1. Plant materials

Tomato plants (Grosse Lisse) susceptible to Fol were used to study the induction of resistance Fol by F1and F4. Tomato seeds were surface disinfected by immersion in 75% ethanol for 1 min followed by extensive rinsing in sterile distilled water. Then seeds were sown in plug trays (plug size 3.4 by 3.4 by 5 cm) containing non-sterile soilless potting mix. Trays were kept in a glasshouse at 25 C, with 16 hr. light and 8 hr. darkness and watered as required.

4.2.2. Tomato plant preparation and treatment

After 25 days of growth of tomato plants, seedlings were uprooted, washed with tap water, and roots were then surface sterilized with 1% sodium hypochlorite for 1 min and 75% ethanol for 30 sec then washed three times with sterilized water to remove residues from ethanol. The seedling was split in two parts with a sterile scalpel from the hypocotyl down to the root system (Amini, 2009; Hibar et al., 2007; Liu et al., 1995). Each half of the root systems was planted in a separate 250 ml tube containing 150 ml of Hoagland nutrient solution. After two days, one side of each split plant was inoculated with 5 ml conidial suspension (10^6 spores/ml) of non-pathogens F1 or F4. Three days after inoculation of the biocontrol F1 and F4, 5 ml conidial suspension (10^6 spores/ml) of pathogens F3445, F5766 or F5768 were inoculated on the non-treated side of the split plant. For controls, one side of each split plant was inoculated with 5 ml conidial suspension (10^6 spores/ml) of non-pathogens F1 or F4 or pathogen separately and the other half was treated with sterile water. All treated tomato plants were grown in a glasshouse at temperature 25 C (day) and 18 C (night). The experiment was a completely randomized design with two replications. When

disease was observed by showing different growth between treatments and control (pathogen only), disease was assessed by measuring differences in growth between treatments and controls. Shoot height was taken from the base of the stem to apex. The shoots and roots were dried in an oven at 70ºC until constant weight, then weighed individually.

4.2.3. Effect of iron on the induction of systemic resistance

Tomato plants with split roots were prepared and inoculated as described above, but with 1 ml conidial suspension (10^6 spores/ml) of non-pathogens F1 or F4 and pathogens 3445 and 5768. The iron nutrition of the nutrient solution was modified by altering the concentration of Fe-EDTA and using Na EDTA to maintain a constant concentration of EDTA. Three levels of iron were prepared. For low iron availability, Na EDTA at 3.72 g/l was substituted for Fe^{3+}-EDTA. For standard iron availability, the quantity of Fe^{3+}- EDTA and Na EDTA was 1.84 and 1.61 g/l respectively. For high iron availability, the quantity of Fe EDTA was 3.68 g/l. The experiment was a completely randomized design with four replicates for each treatment. All treated tomato plants were grown in a glasshouse at temperature 25 C (day) and 18 C (night). Shoot height was taken from the base of the stem to apex. The shoots and roots were dried in an oven at 70ºC until constant weight, then weighed individually (Amini, 2009; Hibar et al., 2007; Liu et al., 1995) (Leeman et al., 1996).

4.2.4. Effect of iron on tomato interaction between pathogen and non-pathogen

Tomato seeds of the cultivar Grosse Lisse were sterilized as described above. Pots 11 cm in diameter and 12 cm high were lined with a clean plastic bag before adding 500 ml of nutrient solution. Seeds were placed on fibreglass flyscreen (1 mm mesh) in clear acrylic tubes inserted in holes in a disc of core flute signboard material that reduced light and evaporation so that the seeds were in contact with the nutrient solution. Plants were allowed to grow for 4 weeks in a glasshouse at 25°C (light) and 18 °C (dark) (Aldahadha et al., 2012). One seedling was grown on the mesh surface in each pot. Three concentrations of iron were prepared as described above. The pots were arranged in a completely randomized design, with four replicates. Inoculation of roots by non-pathogen F4 (2 ml of 10^6 conidia/ ml) was applied at 28 days. Three days after inoculation of the biocontrol F4, 2 ml conidial suspension (10^6 spores/ml) of pathogen F3445 was inoculated. All plants were grown in a glasshouse at temperature 25 C (day) and 18 C (night). Disease was observed by showing different growth

between treatments and control (pathogen only). Shoot height was taken from the base of the stem to apex. The shoots and roots were dried in an oven at 70°C until constant weight then weighed individually.

4.2.5. Statistical analysis

Data were analysed by factorial ANOVA using SPSS version 22. Data were log-transformed when necessary to ensure homogeneity of variance.

4.3. Result

4.3.1. Effect of F1 and F4 on induced resistance to Fusarium wilt

In the split root experiment, pathogens reduced the height of the tomato plants by 37- 43% (Figure 4-1). Non-pathogens did not affect height in the absence of pathogens. There was a significant interaction between pathogen isolate and non-pathogen isolate. The effect of non-pathogens on height differed between pathogen isolates. F1 increased the height of plants inoculated with F5766 and F5768, but not F3445. F4 increased the height of plants inoculated with F3445 and F5766, but not F5768.

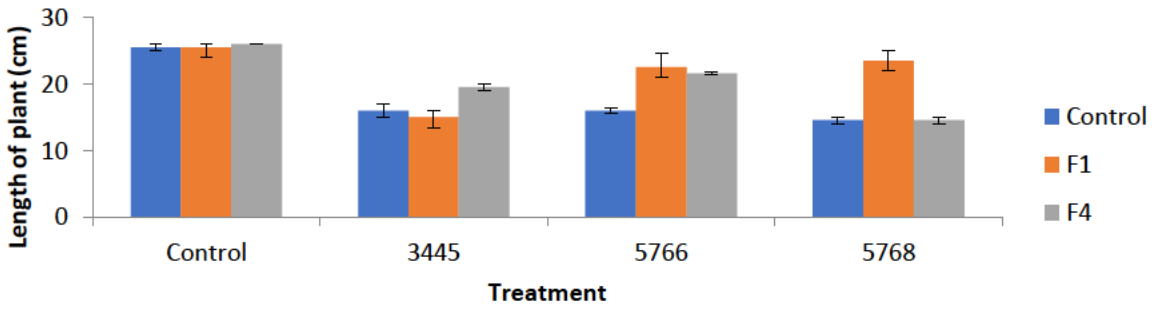


Figure 4-1 Effect of nonpathogenic *F. oxysporum* F1 and F4 on Fusarium wilt of tomato (height) caused by Fol (F3445, F5766 and F5768) in the split-root system. Error bars show standard errors.

Pathogens reduced shoot dry weight of tomato plants by 92- 93% (Figure 4-2). There was a significant interaction between pathogen isolate and non-pathogen isolate. The effect of non-pathogens on shoot dry weight differed between pathogen isolates. F1 increased the shoot dry weight of plants inoculated with F5766 and F5768, but not F3445. F4 increased the shoot dry weight of plants inoculated with F3445 and F5766, but not F5768.

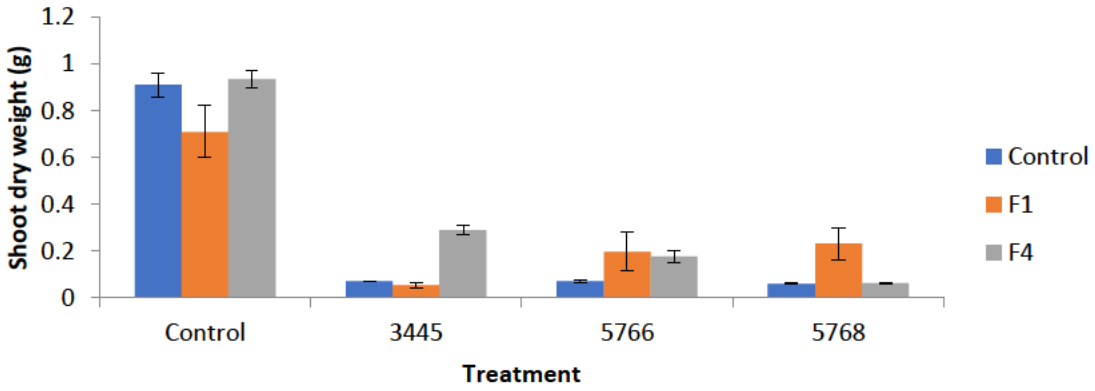


Figure 4-2 Effect of nonpathogenic *F. oxysporum* F1 and F4 on Fusarium wilt of tomato (shoot dry weight) caused by Fol (F3445, F5766 and F5768) in the split-root system. Error bars show standard errors.

Pathogens reduced root dry weight of tomato plants by 85- 89% (Figure 4-3). There was a significant interaction between pathogen isolate and non-pathogen isolate. The effect of non-pathogens on shoot dry weight differed between pathogen isolates. F1 increased the root dry weight of plants inoculated with all pathogens, particularly F3445; however F1 only improved root dry weight of plants inoculated with F5766 and F5768.

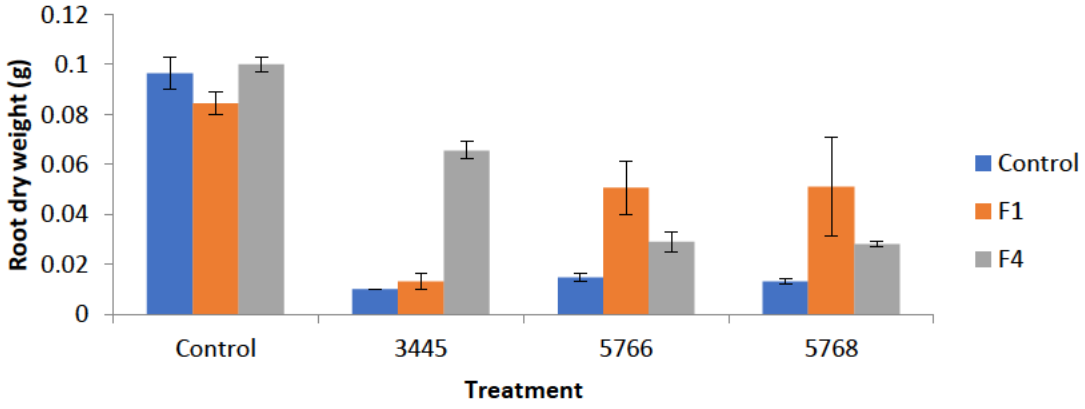


Figure 4-3 Effect of nonpathogenic *F. oxysporum* F1 and F4 on Fusarium wilt of tomato (root dry weight) caused by Fol (F3445, F5766 and F5768) in the split-root system. Error bars show standard errors.

4.3.2. Effect of iron on induction of systemic resistance

There was a highly significant ($P < 0.01$) interaction between pathogen, non-pathogen and iron on the height of tomato plants. Analysis of the data from the experiment using the split root system indicated that iron toxicity increased toward tomato plants with increased concentration. So the highest concentration of iron had strongest negative effect on the height

of tomato plants compared with lowest concentration (Figure 4-4). However, there was a variety of effects of iron on the height of tomato plants in present of non-pathogens. F4 increased the height of tomato plants inoculated with F3445 at standard and double concentrations of iron. F1 increased the height of tomato plants inoculated with F3445 at standard concentration of iron. F1 and F4 both increased height of tomato plants inoculated with F5768 at standard and double concentrations of iron.

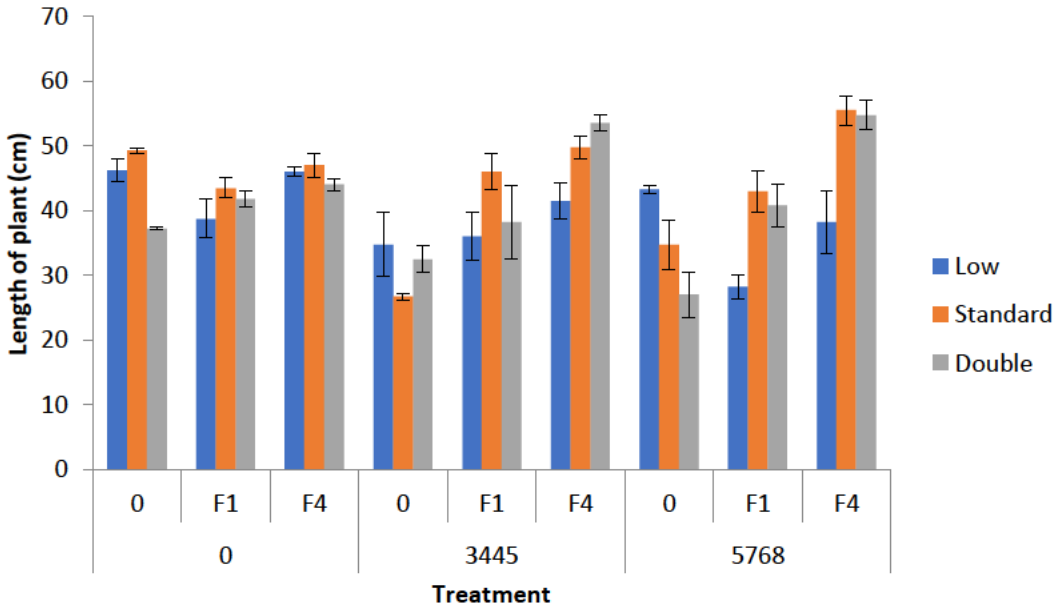


Figure 4-4 Effect of interaction between non-pathogenic F. oxysporum F1 and F4, and concentration of iron, on height of tomato plants infected with Fol, F3445 and F5768 in a split-root system. Pathogen and non-pathogen were inoculated into separate compartments. Error bars show standard errors (n=4)

The effect of iron on the shoot dry weight was similar to that on the height of tomato plants. However, F4 increased shoot dry weight of tomato plants inoculated with F3445 and F5768 at all concentrations of iron while F1 increased shoot dry weight of tomato plants inoculated with F3445 and F5768 at standard and double concentrations of iron (Figure 4-5).

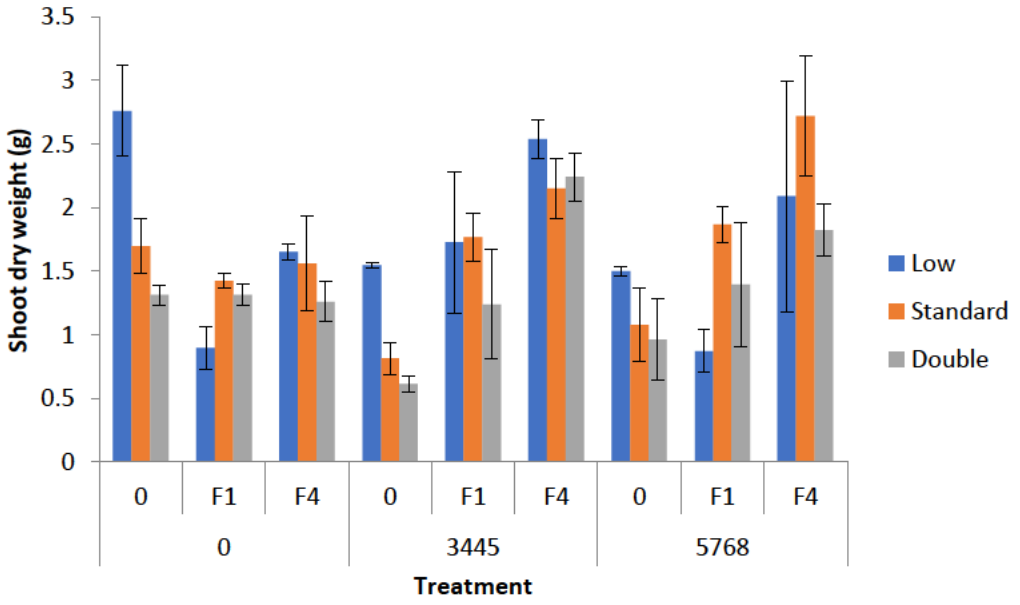


Figure 4-5 . Effect of interaction between non-pathogenic F. oxysporum F1 and F4, and concentration of iron, on shoot dry weight of tomato plants infected with Fol, F3445 and F5768 in a split-root system. Pathogen and non-pathogen were inoculated into separate compartments. Error bars show standard errors (n=4)

The result of root dry weight was the same as shoot dry weight of tomato plants (Figure 4-6).

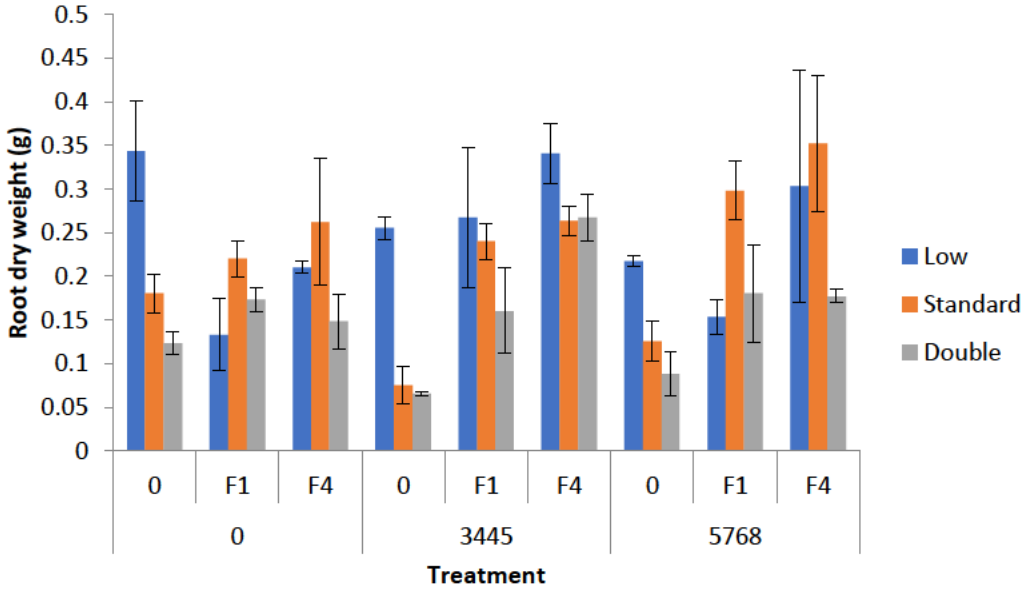


Figure 4-6 Effect of interaction between non-pathogenic F. oxysporum F1 and F4, and concentration of iron, on root dry weight of tomato plants infected with Fol, F3445 and F5768 in a split-root system. Pathogen and non-pathogen were inoculated into separate compartments. Error bars show standard errors (n=4)

4.3.3. Effect of iron on the interaction between pathogen and non-pathogen F4 together

There was a significant effect of iron on the interaction between non-pathogen F4 and pathogen F3445 when they were inoculated into the same nutrient solution. F4 increased the height of tomato plants inoculated with F3445 at all concentrations of iron. However, the proportional increase in height due to F4 was greatest at the lowest level of iron compared with highest level of iron (Figure 4-7).

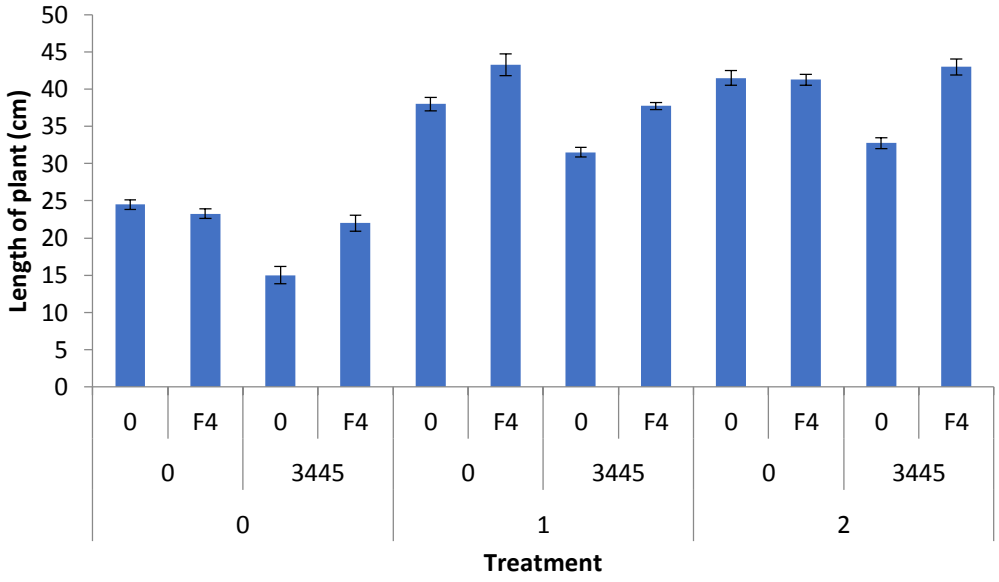


Figure 4-7 Effect of nonpathogenic *F. oxysporum* F4 with different levels of iron on Fusarium wilt of tomato (height) caused by Fol (F3445). Error bars show standard errors. 0 = Low Fe, 1 = Standard Fe, 2 = High Fe

There was a variety of effects of iron on the shoot dry weight of tomato plants inoculated with F3445 in presence of non-pathogen. F4 increased the shoot dry weight of tomato plants inoculated with F3445 at all concentrations of iron. However, the proportional increase in shoot dry weight due to F4 was greatest at the lowest level of iron compared with highest level of iron (Figure 4-8).

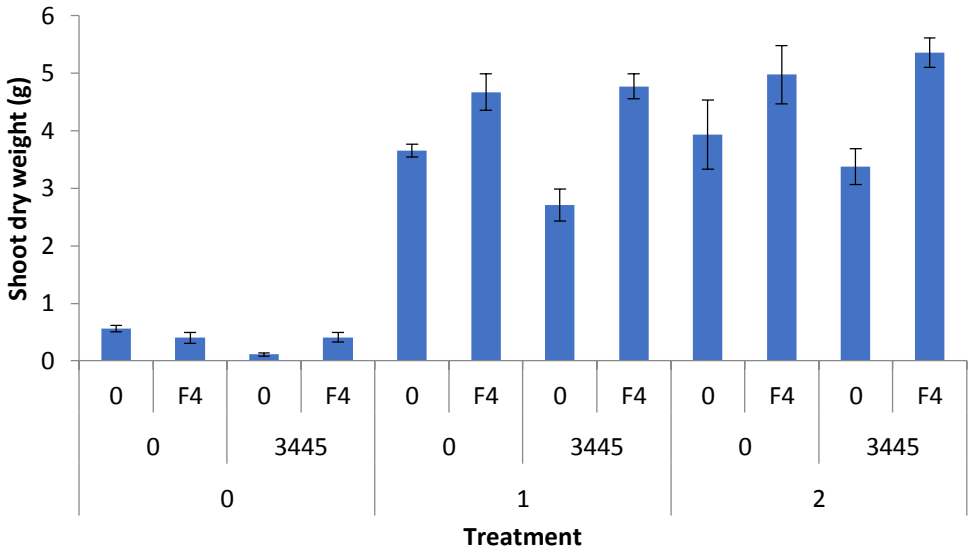


Figure 4-8 Effect of nonpathogenic *F. oxysporum* F4 with different levels of iron on Fusarium wilt of tomato (shoot dry weight) caused by Fol (F3445). Error bars show standard errors. 0 = Low Fe, 1 = Standard Fe, 2 = High Fe

The result of root dry weight was the same as shoot dry weight of tomato plants (Figure 4-9)

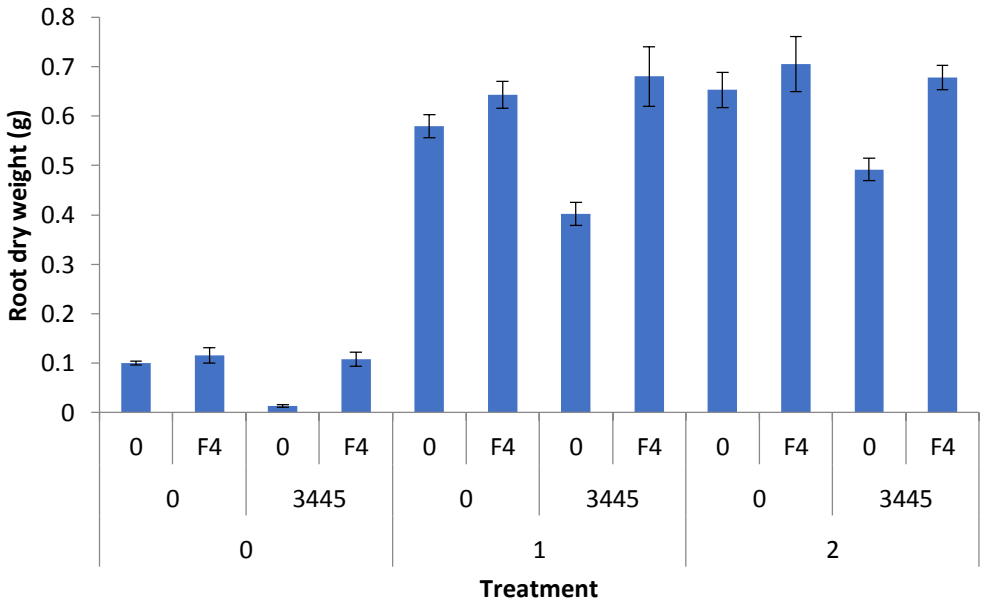


Figure 4-9 Effect of nonpathogenic *F. oxysporum* F4 with different levels of iron on Fusarium wilt of tomato (root dry weight) caused by Fol (F3445). Error bars show standard errors. 0 = Low Fe, 1 = Standard Fe, 2 = High Fe

4.5. Discussion

In this study, two biocontrol isolates were used to evaluate the ability to decrease disease severity after inoculation of the strains in split root systems and non-split root systems. As well, the role of Fe in enhancing induced resistance of tomato plants in the presence of non-pathogens and pathogens was studied. Splitting the root systems in two parts to prevent any direct contact between the pathogen and non-pathogen led to a significant reduction of the disease severity by non-pathogens F1 and F4.

These results are in agreement with previous studies. The plant growth promoting rhizobacteria (PGPR) strains 89B-27(*Pseudomonas putida*) and 90-166 (*Serratia marcescens*) were inoculated on separate halves of roots of cucumber seedlings and planted in separate pots. Both PGPR strains induced systemic resistance against *F. oxysporum f.* sp. *cucumerium* by delaying disease symptom development (Liu et al., 1995). This result strongly supports Hibar et al. (2007) that *T. harzianum* and *T. viride* were able to reduce disease severity of Fol in split root system, when they were applied one week before inoculation with the pathogen.

However, the efficacy of induced resistance varied according to the non-pathogen strain. These results are consistent with those obtained by Olivain et al. (1995) who found that the efficacy of the fungal biocontrol strain was different, with strain Fo47 shown to induce resistance less efficiently than another non-pathogen *F. oxysporum* strain.

So, in the current work, non-pathogens F1 and F4 protected tomato plants from Fusarium wilt, although there was no direct contact with the pathogen. This result indicated that protection did not result from antagonism or competition between F1, F4, and pathogens and shows that non-pathogen F1 and F4 induced resistance to Fusarium wilt in tomato. This could consequently improve the health of the plant or make chemical or physical changes in the plant that could reduce disease incidence. This result supported Tamietti et al. (1993), who found that tomato plants grown in a soil made suppressive by the addition of non-pathogen Fusarium strains showed physiological changes in the stems and leaves which are generally related to systemic induced resistance. Also, non-pathogens could enhance activity of chitinase which is confirmed by (Grenier and Asselin, 1990; Irving and Kuc, 1990; Tamietti et al., 1993; Tuzun et al., 1989) or activity of β-1,3-glucanase or β-1,3-glucosidase (Tamietti et al., 1993).

The induction of systemic resistance at high and standard iron availability by F1 and F4 agrees with observations that strains WCS374 and WCS417 of *P. fluorescences* and their pseudobactin-minus mutant induced systemic resistance against Fusarium wilt of radish at high concentration of iron (Leeman et al., 1996). Also, this result indicated that F1 and F4 reduced the toxicity of iron, by possibly consuming it. This result strongly supports Johnson (2008), who found that siderophores are required for protection against iron-induced toxicity in some fungal organisms. The significant effect of non-pathogen against pathogens at high and standard concentration of iron, could be because the pathogen did not grow well or did not have a big effect on the plant at low concentration of iron, so the pathogenicity was less compared with high concentration of iron. This is supported by Lemanceau et al. (1993), who found that increasing the concentration of Fe enhanced the mycelial growth of *Fusarium oxysporum* f. sp. *dianthi* WCS816, and pathogen WCS816 was more dependent on iron availability for growth than non-pathogen Fo47b10.

The higher overall antagonism at low iron availability by F4 agreed with observations that all the isolates of *Pseudomonas* spp. induced systemic resistance against Fusarium wilt of chickpea when iron availability in the nutrient solution was low (Saikia et al., 2005). Also, the competition between non-pathogens and pathogen for iron led to induced suppressiveness to Fusarium wilt pathogens. This result agrees with the finding by Scher and Baker (1982) that the organization of Fe availability in the infection court, through Fe competition, can induce suppressiveness to Fusarium wilt pathogens. Also, the competition between non-pathogens and pathogen leads to decreased availability of iron in the nutrient solution for pathogens, so that could cause decrease in spore germination of the pathogen. This suggestion is strongly supported by Simeoni et al. (1987) who found that when availability of iron dropped, Fe competition between the two organisms was enhanced and germination of *F.o. f.sp. cucumerinum* was significantly decreased. So non-pathogens F1 and F4 stimulated tomato plants to induced resistance against Fusarium wilt caused by pathogens F3445 and F5768, although, there was no direct contact between them. Also at standard and high concentrations of iron, non-pathogens F1 and F4 reduced disease severity of Fusarium wilt caused by pathogen F3445 compared with low concentrations.

Chapter 5. Effect of root exudate on antagonism

5.1. Introduction

Many studies have investigated the role of plant root exudates in the spore germination, growth and antagonism action of fungi. According to Marschner (1995) root exudates are divided into two kinds of compounds. The first kind is low-molecular weight compounds (amino acids, organic acids, sugar, phenolics, and other secondary metabolites). The second kind is high-molecular weight exudates (polysaccharides and proteins). Organic compounds released by the plant roots activate the microbial activity in the rhizosphere (Bacilio-Jim´enez et al., 2003). Root exudates and their multiple components (e.g. sugars, organic acids, amino acids, and phenolic compounds) play an important role in plant–microbe interactions in the rhizosphere, especially in the initial phase of these interactions (Bais et al., 2006; Bertin et al., 2003; Nelson, 1991). Plant root exudates can supply carbon compounds to stimulate specific microbial populations in the rhizosphere as well as enhance biocontrol efficiency (Morgan et al., 2005). Whipps (2001b) reported that plant pathogens can develop when plant root exudate is applied, resulting in disease. In addition, root exudate had positive influences on the hyphal diameter, length and degree of branching of *Thielaviopsis basicola* (Hood and Shew, 1997).

According to Steinkellner et al. (2008), root exudate of tomato plants plays an important role to increase the microconidial germination rate of biocontrol strain Fo 47 challenged with tomato wilt pathogen Fol 007. Steinkellner et al. (2005) reported that root exudates at different plant ages also varied in their effect on the spore germination of the tomato pathogen *F. oxysporum*. When root exudate of tomato plants was applied, the germination of soil-borne fungus Fol was inhibited (Scheffknecht et al., 2006). Also, in the vicinity of tomato roots where exudates would occur, there was a big diversity in mycelial development between strains of *F. oxysporum* (Steinberg et al., 1999).

Tomato root exudates supply the great energy source in the root zone for microbial activity such as organic acid and sugar (Kravchenko et al., 2003). It was demonstrated previously that citric acid was the biggest organic acid in root exudates. However, malic and succinic acid were very important, but the levels of both of them were very dependent on the plant age (Kamilova et al., 2006). As well as, in the exudate of tomato root, the major sugar

compounds were glucose, fructose and xylose (Kamilova et al., 2006). So, the main objectives of this chapter were to assess the effect of root exudate and components such as sugar, organic acid, and amino acid of tomato plants on the spore germination, growth and antagonism action of fungi in laboratory and glasshouse experiments.

5.2. Materials and Methods

5.2.1. Root exudate production

Root exudates from tomato plants were produced following the method described by Shang et al. (2000). Seeds of tomato cultivar Grosse Lisse were rinsed for 3 min with 1% sodium hypochlorite for surface disinfestation. The seeds were washed three times with sterilized distilled water. Then 10 tomato seeds were placed in plastic tubes (150 ml), containing 20 mL of sterile Hoagland nutrient solution. The tubes were kept in a growth chamber at 25 C with 16 h light and 8 h dark. The nutrient solution containing root exudates was collected after 21 days. For checking microbial contamination, one millilitre was taken from the root exudate and cultured on PDA medium and incubated at 25 C for 5 days. The solution was stored frozen until used in experiments.

For the first series of experiments, four liquid media were used. These were root exudate in Hoagland nutrient solution, Hoagland nutrient solution, Hoagland nutrient solution plus sucrose at concentration 1.667 g/l or water (Tahat et al., 2010). The concentration of sucrose was chosen because it was typical of the concentration of soluble C found in the rhizosphere of plants (Cheng et al., 1993)

5.2.2. Effect of root exudate on the germination of fungal spores

The germination assay was achieved in sterile 96 well microplates. Aliquots of 200 µl of the four liquid media were mixed with 1 µl of approximately 10^6 conidia/ml spore suspension of F1, F2, F3, F4, F5, F6, F7, and F5769 separately and incubated at 25°C in the dark. Relative microconidia germination and growth was determined after 48 h by measuring absorbance in a microplate reader at 600 nm.

5.2.3. Effect of root exudate on antagonism in vitro

All seven antagonistic *F. oxysporum* isolates were used to test the effect of root exudates on antagonism. 20 ml of the four liquid media were placed in Petri dishes. Molten water agar was added to each Petri dish and mixed to give a final concentration of 20 g/l agar. Both the antagonist and the pathogen F5769 were simultaneously inoculated at the opposite ends 5 cm apart from each other with mycelial plugs of 5 mm diam. from the margin of 6 days old cultures. In controls the pathogen was alone plated on one side of the plate at the periphery. Inoculated plates were incubated at 25 °C for 7 to 10 days. The experiment was replicated three times for each treatment. Results were expressed as means of radial fungal growth and % inhibition of fungal growth of the pathogen (Ramezani, 2010; Subhani et al., 2013; Whipps, 1987).

5.2.4. Effect of root exudate on fungal growth

Four liquid medium were used as above in 150 ml plastic tubes. Each tube had 20 ml filter-sterilized liquid medium and 1 ml of approximately 10^6 conidia/ml spore suspension for non-pathogens F1, F2, F3, F4, F5, F6, F7 and pathogen F5769 separately. After inoculation, tubes were incubated for 6 days at 25 °C in a rotary shaker at 120 rpm. There were three replicated for each treatment. Mycelial growth of each of the test isolates was harvested in Miracloth, oven dried at 60 °C and weighed.

5.2.5. Effect of organic acid and sugar on antagonism in vitro

The effect of organic acids (malic, succinic and citric acid) and sugars (lactose, fructose, glucose and xylose) on the antagonism between non-pathogens (F4, F1) and pathogen F5769, was studied in dual culture on Czapek Dox agar medium. The organic acids and sugars were used in place of the sucrose in Czapek Dox agar medium with five concentrations (5, 10, 20, 25, 30 g/l). The dual culture plates and controls were inoculated as described previously. Inoculated Petri dishes were incubated at 25 °C for 7 to 10 days. There were three replicated for each treatment. Results were expressed as means of radial of fungal growth and % inhibition of fungal growth of the pathogen.

The effects of low concentrations of organic acids and sugars on antagonism between non-pathogen F4 and pathogen F5769 was studied in a modification of the above experiment. The organic acids and sugars were used in place of the sucrose in Czapek Dox agar medium with

two low concentrations (0.5 and 2 g/l). The dual culture plates and controls were inoculated and incubated as described previously.

5.2.6. Effect of organic acids and sugars on antibiotic production of non-pathogen

Non-pathogens F1 and F4 were grown in liquid Czapek Dox medium containing sugars (lactose, fructose, xylose and glucose) or organic acids (citric, succinic and malic acid) at concentrations of 5, 10, 20, 25, 30 g/l in place of sucrose. Five 5-mm discs of 6 day old non-pathogen culture was transferred to each of 500 ml flasks contain 250 ml of medium and incubated at 25 C on a rotary shaker at 120 rpm for 21 days. The mycelial biomasses were filtered through Miracloth and filter paper (Whatman grade 41) and then culture filtrate was filtered through a 0.45 µm membrane. 5 ml of culture filtrate was placed in the sterilized Petri-dishes (9 cm^3) and then 15 ml ¼ PDA was added and mixed carefully. Five mm disc from 6 days old culture of pathogen was used for inoculation. The inoculated Petri dishes were incubated at 25 °C. Mycelial growth of the pathogen was measured on each Petri dish. The control was pathogen growth in Petri dishes containing ¼ PDA plus culture filtrate from the pathogen. There were three replicated for each treatment. Radial growth of the pathogen was measured and compared to control growth (Chen et al., 2012; Schalchli et al., 2012; Whipps, 1987).

5.2.7. Effect of root exudate on antagonism in planta

Tomato seeds of the cultivar Grosse Lisse were planted in plug trays (plug size 3.4 by 3.4 by 5 cm, 6 plugs/tray) containing non sterile soilless potting mix. After 2 weeks tomato plants were transplanted into 17 cm diameter pots containing non sterile soilless potting mix. Fungi (F1, F4 and F5768) were grown in potato dextrose broth for 7 days on a rotary shaker at 120 rpm at 25 °C, blended, and propagule counts determined on a haemocytometer. After one week conidial suspensions of non-pathogenic (F1 and F4) *F. oxysporum* (200 ml of approximately 10^6 conidia/ml) were added to each pot containing 2 kg of potting mix and then after two days the conidial suspensions of pathogenic F5768 (200 ml of approximately 10^6 conidia/ml) was added to each pot. Sugar (lactose and fructose) and organic acid (malic and succinic) solutions were prepared. The concentrations of lactose, fructose, malic and succinic were 0.171, 0.180, 0.134 and 0.118 g/l respectively. This was equivalent to 1 mM of each compound except for the disaccharide lactose, which was 0.5 mM. 300ml from each

solution was added to the pots weekly. Each treatment consisted of four replicate pots of 1 plant /pot. Disease was observed by showing different growth between treatments and control (pathogen only). After 6 weeks, height, shoot and root dry weights were determined. Shoot height was taken from the base of the stem to apex. The shoots and roots were dried in an oven at 70 °C until constant weight, then weighed individually (Larkin and Fravel, 2002; Scher and Baker, 1982)

5.3. Results

5.3.1. Influence of root exudate on spore germination

There were significant effects of fungus and of medium on spore germination, but the interaction between these was not significant. F5 and F6 had the highest absorbance, while F4 and F7 had the lowest (Figure 5-1). Germination was higher in Hoaglands solution and Hoaglands plus exudate than in water, but did not differ between these two media (Figure 5-2). Addition of sucrose to Hoaglands solution increased germination.

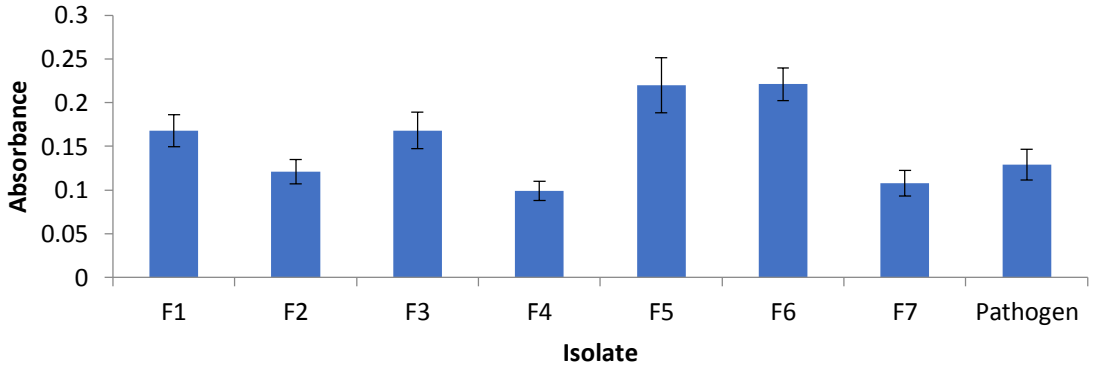


Figure 5-1 Spore germination of pathogen and non-pathogen *Fusarium spp.* Data are mean absorbance of germling suspensions in four media. Error bars show standard errors (n = 12).

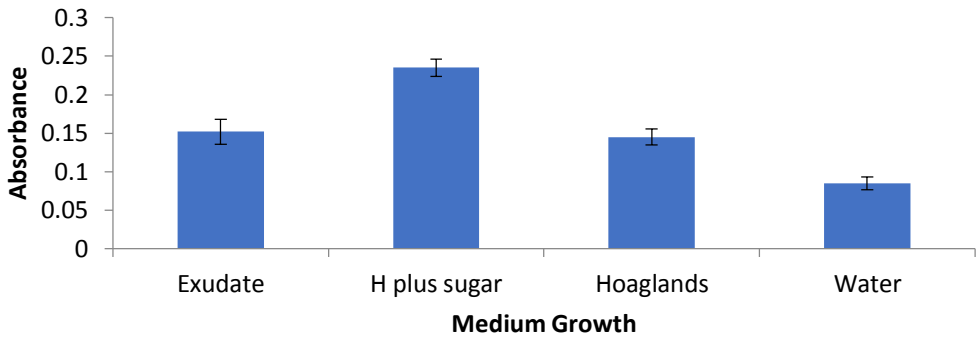


Figure 5-2 Effect of root exudate, Hoagland nutrient solution, Hoagland nutrient solution plus sugar and water on germination of spores of *Fusarium spp.* Error bars show standard errors (n = 12).

5.3.2. Effect of root exudates on antagonism in vitro

There was a significant effect of all treatments on the percentage growth inhibition for the pathogen (Table 5-1). Root exudates increased the inhibition of growth of the pathogen for all antagonists, while the Hoagland nutrient solution plus sugar decreased the inhibition of growth of the pathogen relative to Hoagland solution by itself. Treatments F4 and F1 with root exudates showed higher inhibition of growth of the pathogen compared with other fungi.

Table 5-1 Percentage inhibition of radial growth of pathogen F5769 in dual culture with antagonist on Hoagland solution plus agar. The least significant difference for comparing data within a row is 3.44.

Fungi	Root exudate plus Hoagland Nutrient solution	Hoagland Nutrient solution	Sugar plus Hoagland Nutrient solution
F1	37.16	26.67	18.85
F2	33.63	26.67	17.91
F3	32.75	27.5	18.85
F4	43.37	25.82	16.98
F5	33.63	29.17	18.85
F6	31.85	27.5	21.68
F7	32.04	23.32	15.08

5.3.3. Effect of root exudates on fungal growth

Root exudates increased the weight of fungi compared to Hoagland nutrient solution while the highest weights were in Hoagland solution with sucrose (Table 5-2).

Table 5-2 Effect of three liquid medium (root exudate plus Hoagland nutrient solation, Hoagland nutrient solation and Hoagland nutrient solation plus sugar on the dry weight (mg) of Fusarium spp. The least significant difference for comparing data within a row is 2.04

Fungi	Root exudate plus Hoagland Nutrient solution	Hoagland Nutrient solution	Sugar plus Hoagland Nutrient solution
F1	12.33	8	18.33
F2	12	6.67	24.67
F3	10.67	7	17.67
F4	8	7.33	23
F5	15	13.67	23.33
F6	16	13	20.67
F7	16.33	12.33	23.67
5769	14.67	10	21.67

5.3.4. Effect of organic acid on antagonism in vitro

The inhibition of growth of pathogen F5769 by F1 decreased with increasing concentration of malic acid from 33.5% at the lowest concentration to 16.2% at the highest concentration However, the inhibition of growth of the pathogen by F4 increased with increasing concentration of malic acid with F4 (Figure 5-3). Also, F4 had a larger effect on the mycelial growth of pathogen F5769 compared to F1, in the presence of malic acid in dual culture

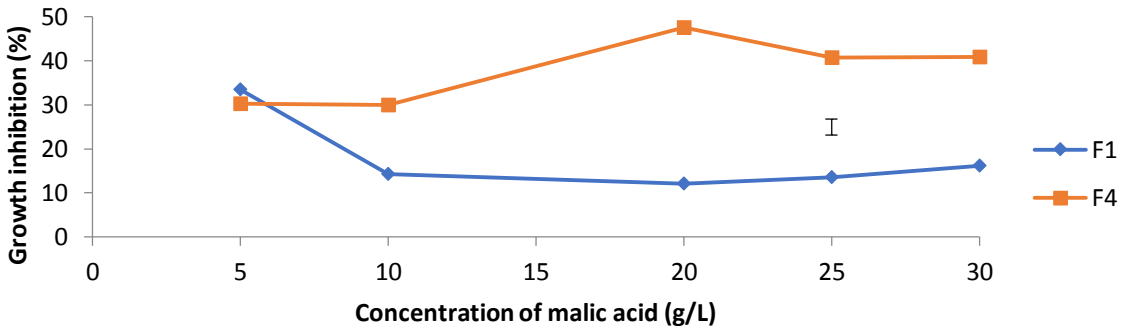


Figure 5-3 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of malic acid as sole organic source. Error bar shows standard error for comparing any two data points (n=3).

Succinic acid had a significant effect on the inhibitory action of F1 and F4 against pathogen 5769. The inhibition of growth of the pathogen by F1 and F4 decreased with increasing

concentration of succinic acid (Figure 5-4). Also, the inhibitory effect of F1 on the mycelial growth of pathogen 5769 was greater than F4 in the presence of succinic acid

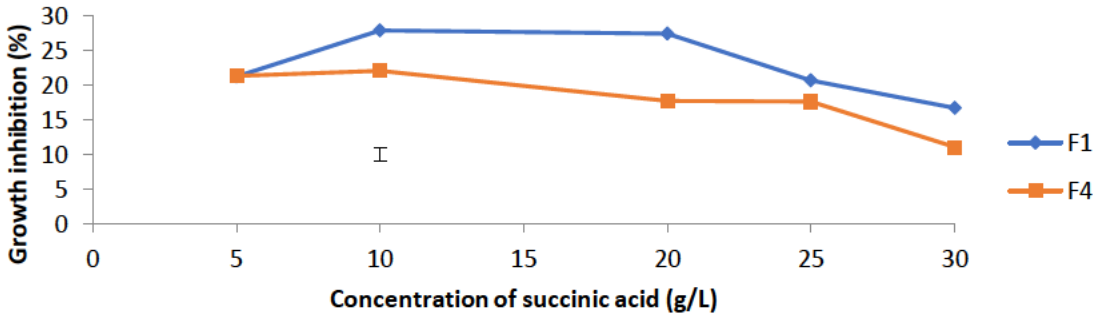


Figure 5-4 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of succinic acid as sole organic source. Error bar shows standard error for comparing any two data points (n=3).

As the concentration of citric acid increased, inhibition by F1 and F4 decreased slightly to a minimum at 20 g/l (Figure 5-5). Maximum inhibition occurred at the highest concentrations of citric acid. F4 was slightly more inhibitory than F1 on citric acid.

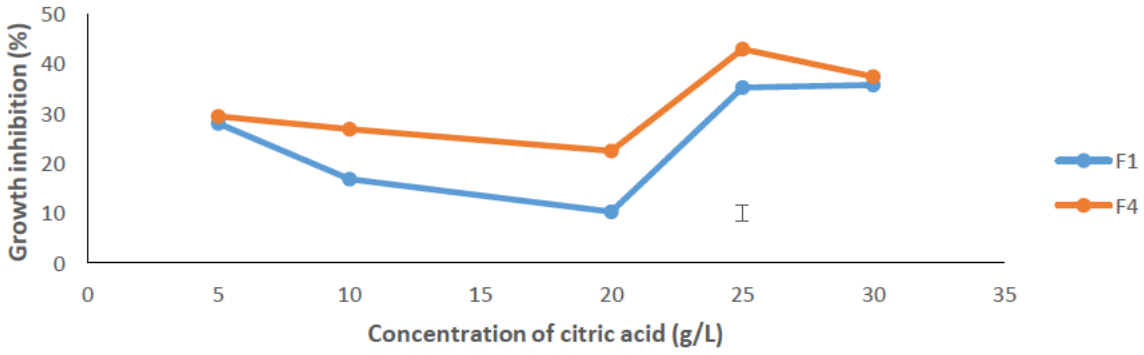


Figure 5-5 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of citric acid as sole organic source. Error bar shows standard error for comparing any two data points (n=3).

In low concentrations of organic acids (0.5 and 2.0 g/L), there was no significant effect on inhibition of growth of the pathogen.

5.3.5. Effect of sugar on antagonism in vitro

Inhibition by F1 was slightly higher at 20 and 25 g/l than other concentrations of lactose (Figure 5-6). F4 showed no inhibition at the lowest concentrations of lactose (5 and 10 g/l) and highest inhibition was at the higher concentrations.

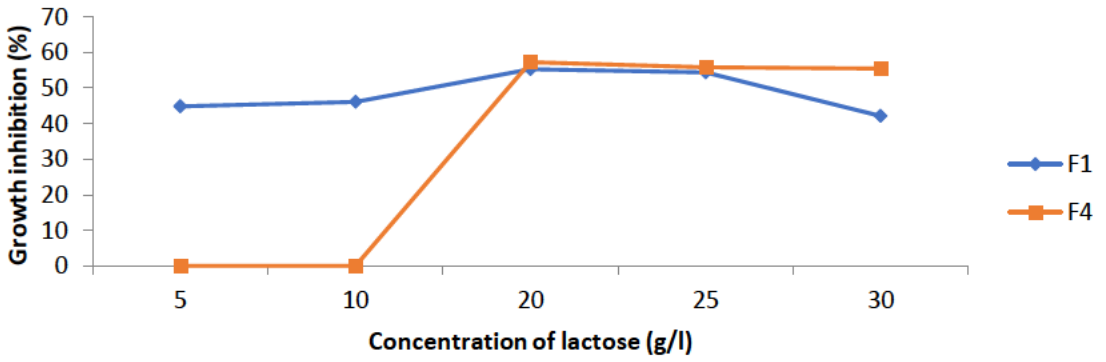


Figure 5-6 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of lactose as sole carbon source. Standard error is too small to show (n=3).

Inhibition by F1 was slightly higher at lowest concentration of fructose 5 g/l than other concentrations (Figure 5-7). Maximum inhibition by F4 occurred at 25 g/l of fructose. F4 was slightly more inhibitory than F1 on fructose.

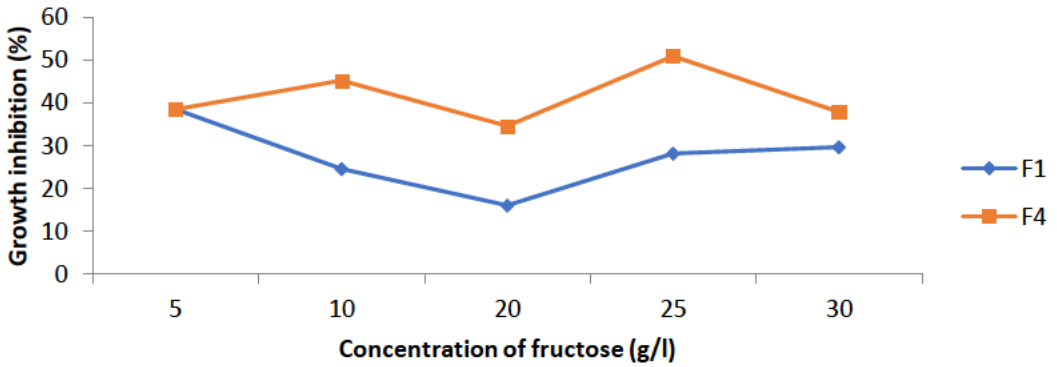


Figure 5-7 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of fructose as sole carbon source. Standard error is too small to show (n=3)

Glucose had a significant effect on the inhibitory action of F1 and F4 against pathogen F5769. As the concentration of glucose increased, inhibition by F1 increased slightly to a maximum at 30 g/L while, the inhibition of growth of pathogen by F4 decreased with increased concentration of glucose (Figure 5-8).

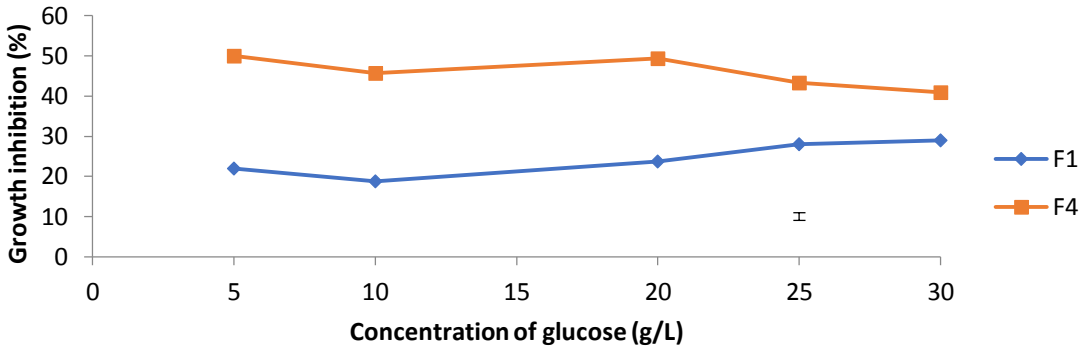


Figure 5-8 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of glucose as sole carbon source. Error bar shows standard error for comparing any two data points (n=3).

Xylose had a significant effect on the inhibitory action of F1 and F4 against pathogen F5769. As the concentration of xylose increased, the inhibition of growth of pathogen by F4 increased. Inhibition by F1 decreased slightly to a minimum at 20 g/l, as the concentration of xylose increased (Figure 5-9). F4 had a larger effect on the mycelial growth of pathogen 5769 compared to F1, in the presence of xylose in dual culture.

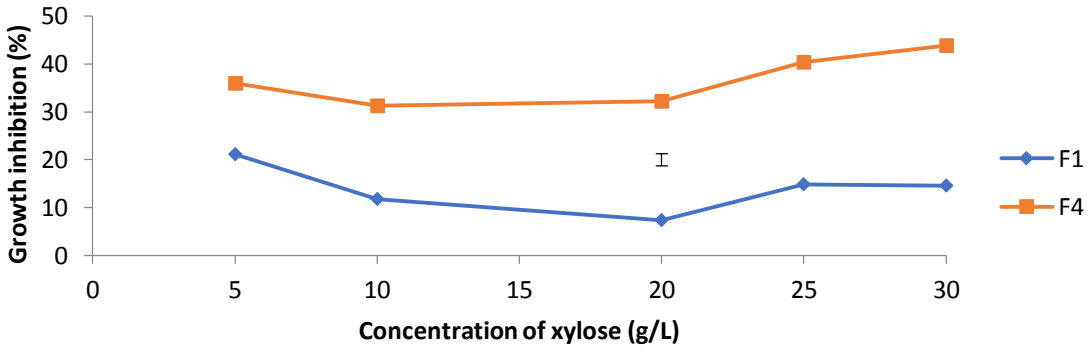


Figure 5-9 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of xylose as sole carbon source. Error bar shows standard error for comparing any two data points (n=3).

In low concentrations of sugars (0.5 and 2.0g/L), there was no effect on antagonism between antagonist and pathogen

5.3.6. Effect of organic acids and sugars on antibiotic production of non-pathogen

Growth of the pathogen was inhibited by filtrate of F4 from all concentrations of fructose except 20 g/l (Figure 5-10). Inhibition was greatest at the highest and lowest concentrations.

Growth of the pathogen was promoted by filtrate of F1 from the lowest concentration of fructose, and showed only slight inhibition or promotion at other concentrations.

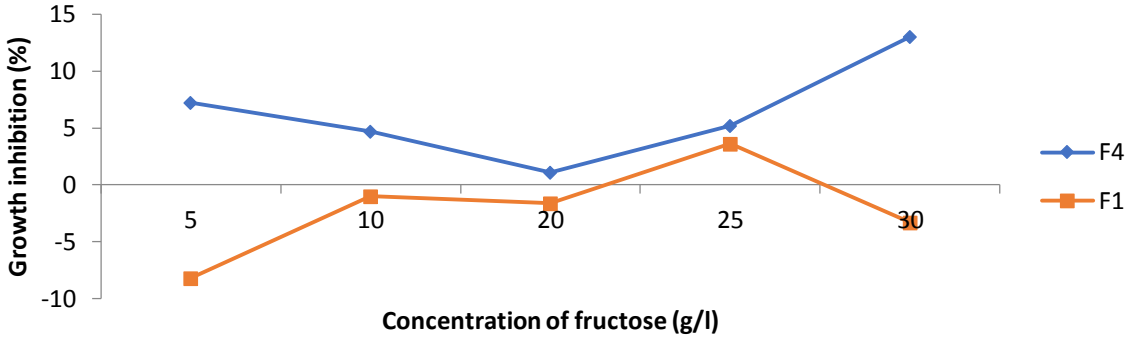


Figure 5-10 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of frutose as sole carbon source. Standard error is too small to show (n=3).

Concentration of lactose had a significant effect on action of filtrate of F1 and F4. Inhibition of growth of pathogen by filtrate of F4 was greatest at 20 and 25 g/l, whereas inhibition of growth of pathogen by filtrate of F1 was greatest at 20 g/l (Figure 5-11). As concentration of lactose decreased or increased from 20 g/l, the inhibition of growth of pathogen was decreased. Filtrate of F4 was slightly more inhibitory than filtrate of F1 on lactose.

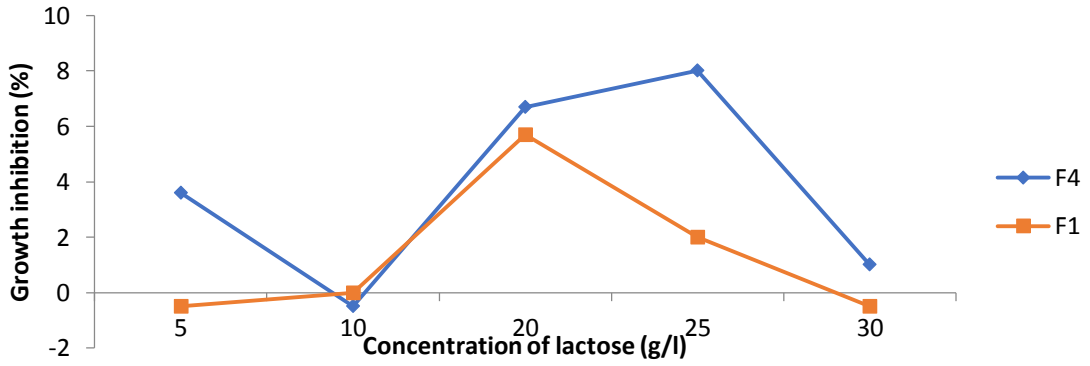


Figure 5-11 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of lactose as sole carbon source. Standard error is too small to show (n=3).

Growth of the pathogen was promoted by filtrate of F4 from the lowest concentration of glucose 5 and 10 g/l (Figure 5-12). However, the inhibition of growth of pathogen by filtrate of F4 was greatest at 20 g/l of glucose. Filtrate of F1 promoted growth of pathogen from all concentration of glucose except 30 g/l. Filtrate of F4 was slightly more inhibitory than filtrate of F1 on glucose.

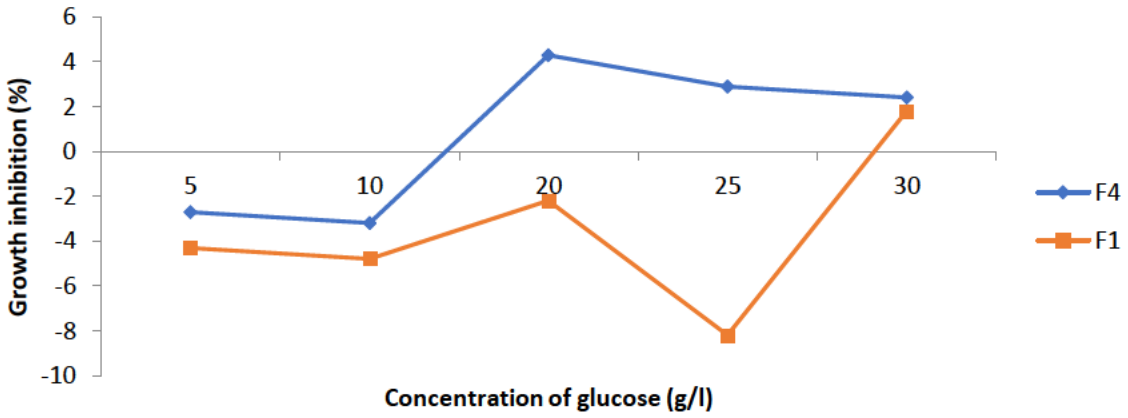


Figure 5-12 Inhibition of growth of Fol isolate F5679 by filtarte of F1 and F4 on ¼ PDA with a range of concentrations of glucose as sole carbon source. Standard error is too small to show (n=3).

Filtrate of F4 inhibited the growth of pathogen at concentration 25 g/l, whereas other concentrations promoted growth of pathogen (Figure 5-13). Culture filtrate of F1 promoted growth of the pathogen at all concentrations of xylose

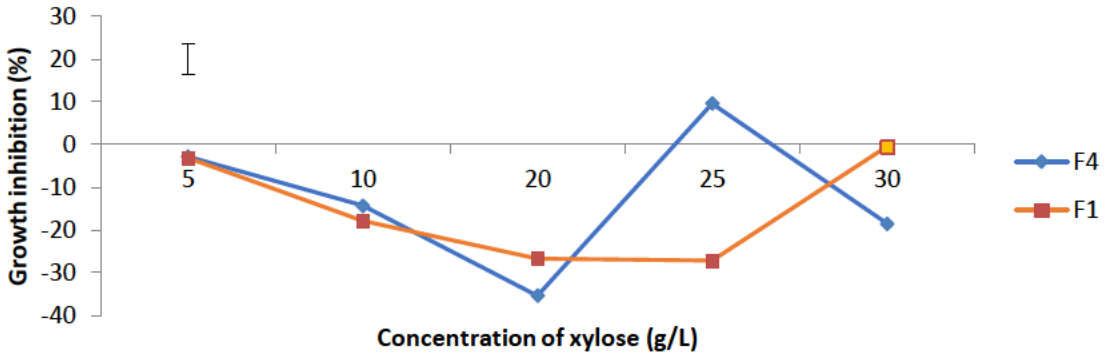


Figure 5-13 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of xylose as sole carbon source. Error bar shows standard error for comparing any two data points (n=3).

As the concentration of malic acid increased, inhibition of growth of pathogen by filtrate of F1 increased (Figure 5-14). The biggest inhibition of growth of pathogen by filtrate of F4 was at 20 and 25 g/l of malic acid. Filtrate of F4 was more inhibitory than filtrate of F1 on malic acid except at 30 g/l (Figure 5-14).

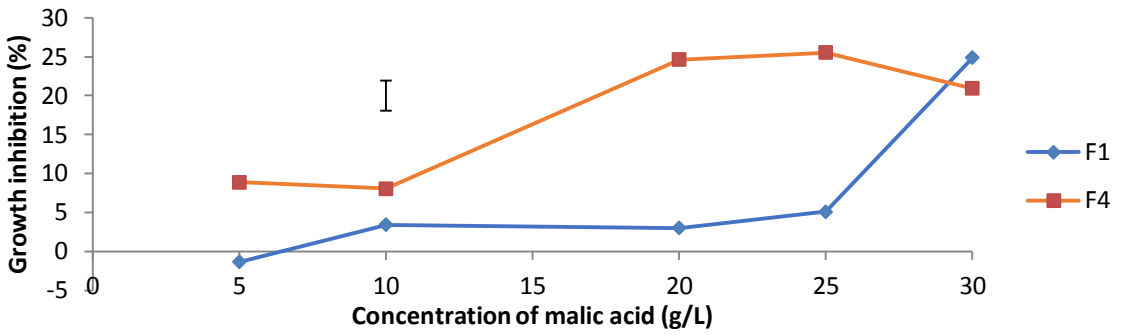


Figure 5-14 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of malic as sole orgainc source. Error bar shows standard error for comparing any two data points (n=3).

As the concentration of succinic acid increased, inhibition by F1 and F4 decreased slightly to a minimum at 10 g/l (Figure 5-15). Maximum inhibition occurred at the highest concentrations 30 g/l of succinic acid. F4 was more inhibitory than F1 on succinic acid.

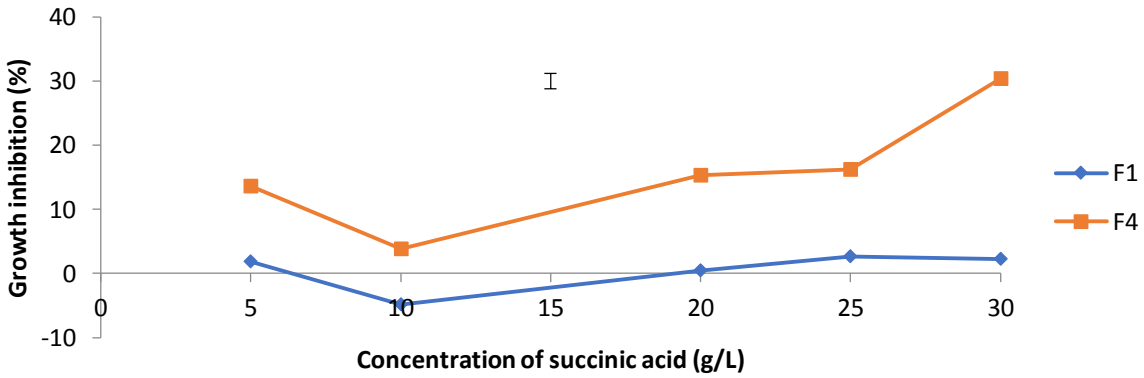


Figure 5-15 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of succinic as sole orgainc source. Error bar shows standard error for comparing any two data points (n=3).

Inhibition of growth of pathogen by filtrate of F4 was greatest at the lowest concentrations of citric acid 5 g/l, whereas inhibition by filtrate of F4 decreased slightly to a minimum at 10 g/l. The inhibition of growth of pathogen by filtrate of F1 was greatest at highest concentration of citric acid 30 g/l (Figure 5-16).

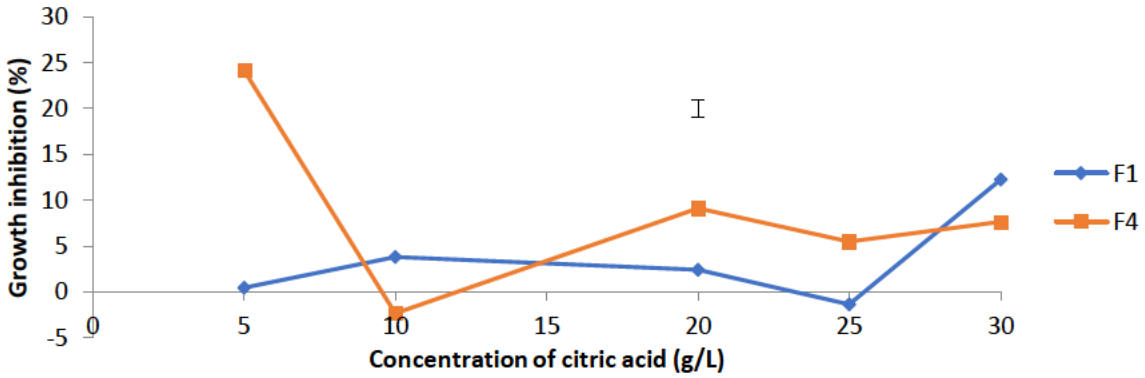


Figure 5-16 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of citric as sole orgainc source. Error bar shows standard error for comparing any two data points (n=3).

5.3.7. Effect of root exudate on antagonism in planta

There was a significant effect of antagonists F1 and F4 on the height of tomato plants compared with pathogen only. Both F1 and F4 increased plant height compared with the control (Figure 5-17). There were no significant effects of root exudate components (sugar and organic acid) or significant interactions between exudate components and the effect of antagonists F1 and F4 on the height of plants.

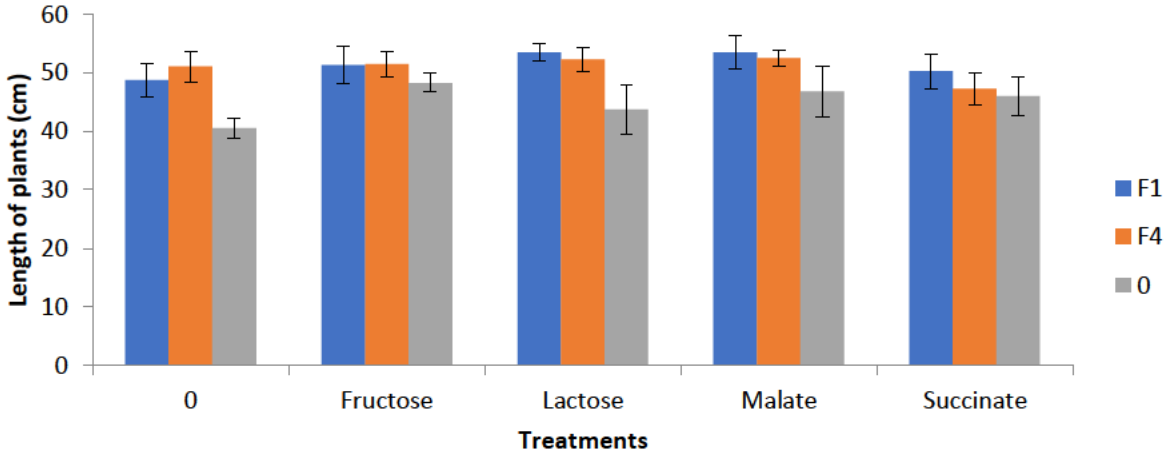


Figure 5-17 Effect of sugars and organic acids (300ml/2kg soil) on height of tomato plants when introduced into soil infested with pathogen F5768 with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=4).

Both antagonists significantly increased shoot and root dry weight of tomato plants infected with the pathogen (Figure 5-18). All treatments of root exudate components (sugar and organic acid) increased shoot and root dry weight compared with untreated plant. Malic acid was recorded the highest rates of shoot and root dry weight compared with other treatments.

However, there was no significant interaction between antagonist fungi and root exudate components (sugar and organic acid) on the shoot and root dry weight.

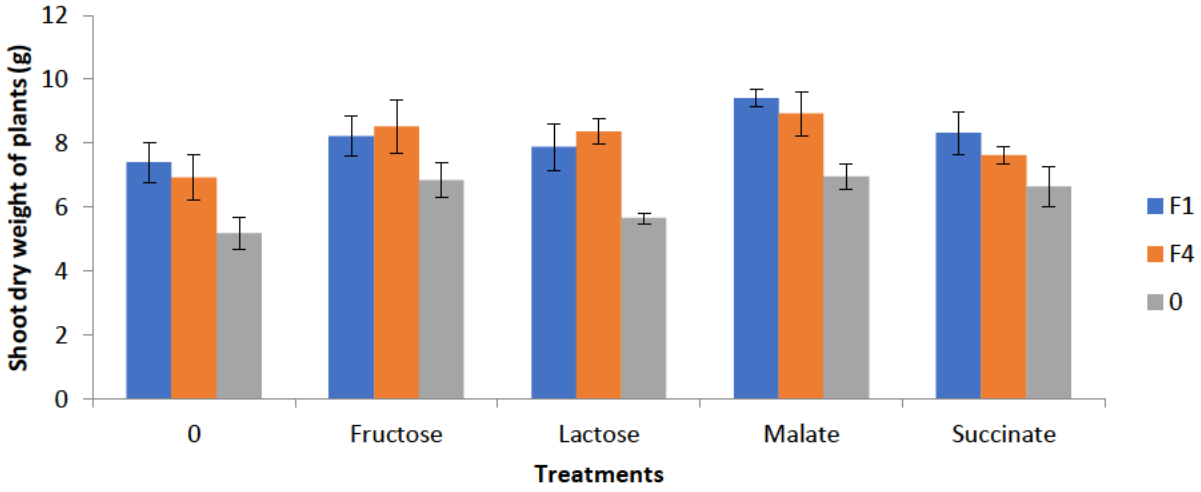


Figure 5-18 Effect of sugars and organic acids (300ml/2kg soil) on shoot dry weight of tomato plants when introduced into soil infested with pathogen F5768 with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=4).

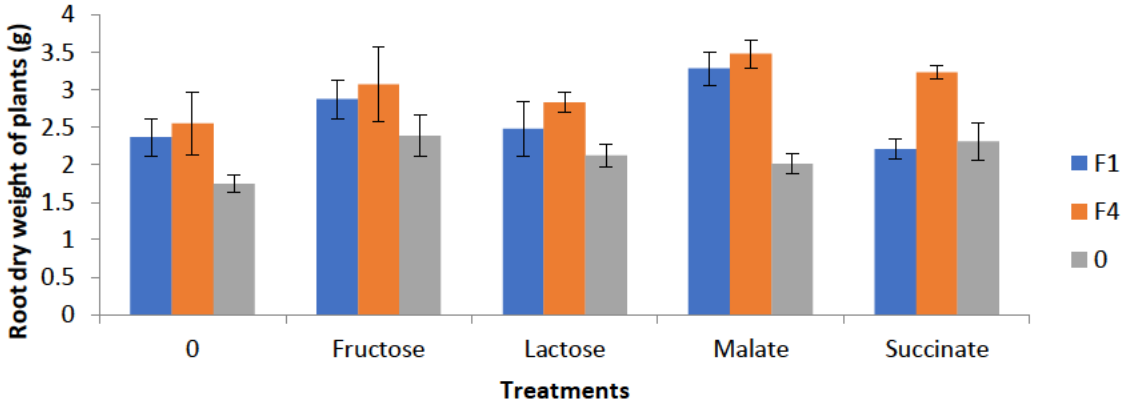


Figure 5-19 Effect of sugars and organic acids (300ml/2kg soil) on root dry weight of tomato plants when introduced into soil infested with pathogen F5768 with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=4).

5.4. Discussion

The stimulation of microconidial germination of tomato pathogens and non-pathogens by root exudate was found, confirming pervious finding showing that tomato root exudate stimulated the microconidia of two tomato pathogens Fol 007 and FORL101587 and other *F. oxysporum* strains (Steinkellner et al., 2005; Steinkellner et al., 2008). In the present study, the germination of pathogen and non-pathogen was increased in the presence of root exudate, compared to water only. This may be because root exudate contains sugars, amino acids and organic acids which are known as general germination stimuli of spores (Nelson, 1991).

Root exudates played an important role in inhibition of growth of pathogen F5769 by all antagonists. These results could be because root exudate has compounds such as sugar, organic acid, amino acid and others that have effects on interactions between the pathogen and non- pathogens. These results are consistent with those obtained by Bais et al. (2006); Bertin et al. (2003); Nelson (1991), who observed that root exudates and their multiple components (e.g. sugars, organic acids, amino acids, and phenolic compounds) play an important role in plant–microbe interactions in the rhizosphere, especially in the initial phase of these interactions. These compounds of root exudates also could stimulate antagonists to produce antibiotics which inhibit growth of pathogens. Buxton (1960) found that pea root exudate increased the ability of ten prevalent rhizosphere fungi to inhibit growth of the pathogen *F. oxysporum* f.sp. *pisi.*

Also, root exudate enhanced growth of fungi and this result was most likely due to the sugars in root exudate because the highest weights were found in Hoagland solution with sucrose. This result confirmed what Kravchenko et al. (2003) found that rhizobacteria showed good growth on a mixture of organic acids and sugars which is typical of the proportion of these substances in tomato root exudate. Amino acids could also play an important role to increase growth of fungi. This result agrees with those obtained by Simons et al. (1997), who observed that amino acids in tomato root exudate assisted bacteria to colonize the root tip.

There were various effects of organic acids on the antagonistic action of F1 and F4 against mycelial growth of pathogen 5769. This result could be due to the organic acids acting as carbon sources which enhanced growth of fungi or could increase competition between fungi for nutrients. This result strongly supports the theory of Kamilova et al. (2006); Kamilova et al. (2005) that the major exudate carbon source, citric acid enhanced growth of bacteria in the

rhizoshere and competition for nutrient. Also, Chin-A-Woeng et al. (2000); Kamilova et al. (2005) reported that organic acid usage by rhizobacteria is decisive for competitive tomato root tip colonization, a process which is predominating essential for biocontrol.

Also, sugar had various effects on the antagonistic action of F1 and F4 against mycelial growth of pathogen 5769. Sugars were used as sole carbon source. So some sugar compounds assisted the non-pathogen to compete with the pathogen for growth. In the presence of sugar compounds, the competition between non-pathogens and pathogen for the carbon source increased. These results confirmed those of Kravchenko et al. (2003), who found sugar plays an important role in antifungal activity of rhizobacteria in rhizosphere of plant.

So, organic acids and sugar separately had some influence on the antagonism between non-pathogen F1, F4 and pathogen. These results could be due to sugar or organic acid when used separately not being enough to enhance antagonist of non-pathogen against pathogen. These results are consistent with those obtained by Lugtenberg et al. (1999). They observed that *P. fluorescens* strain WCS365 and OE28 are very good users of sugars but poor colonizers of tomato roots, therefore the strain must use another major carbon source for colonization such as organic acids.

There was a different effect of sugar and organic acid on the antibiotic production of non-pathogen F1 and F4 which it was using against the pathogen. Some of the sugar (fructose, lactose and glucose) and organic (malic, succinic and citric acids) compounds stimulated the non-pathogens to produce antibiotic against the pathogen. These results are consistent with those obtained by Kravchenko et al. (2003). They believed that the antifungal activity of rhizobacteria introduced into the plant rhizosphere depends on the sugar and organic acid composition of the root exudates of these tomato plants. Also this result is confirmed by Duchesne et al. (1988) who observed that *Pinus resinosa* root exudate from seedlings inoculated with *P. involutus* led to increase in fungitoxic activity against Fusarium root. This result was due to plant factors that control the genetic regulation of biosynthetic pathways in *P. involutus* leading to antibiotic biosynthesis.

Plant root exudate can supply carbon compounds to activate specific microbial populations in the rhizosphere and improved biocontrol efficiency (Morgan et al., 2005). However, plant root exudate could also cause the development of plant pathogens, resulting in disease (Whipps, 2001b). In the present study, adding sugar or organic acids to soil did not have an

effect on the interaction between non-pathogen and pathogen. This result could be due to sugar or organic acid separately improving spore germination for both non-pathogens and pathogen. This result strongly supports the finding of Steinkellner et al. (2005) that the root exudate stimulate microcondia germination of Fol. As well, sugars are recognized to be stimulatory to germination of *Fusarium spp*. (Nelson, 1991). Therefore the non-pathogen was less suppressive towards the pathogen in pot trials. So most of the components of root exudates such as sugar, organic and amino acids showed the ability to the improved antagonist of non-pathogens against the pathogen in vitro. However, there was a little effect of these components on the antagonist in pots trial

Chapter 6. Effect of nutrients on antagonism

6.1. Introduction

Previous studies have referred to the role of nitrogen and iron in biological control of soil borne plant pathogens by competition for nutrients (Benson and Baker, 1970; Cook and Schroth, 1965). The pathogen reacted to a wide range of nutritional sources with large variation in its utilization

Nitrogen is considered an important component required for protein synthesis and other vital functions (Khilare and Ahmed 2011). The availability of nitrogen, but not carbon, restricted the activities of antagonists of *Fusarium pseudograminearum* in straw (Singh Lakhesar et al., 2010). In addition, the form of nitrogen can impact the rate of displacement and mortality of the pathogen in host residues (Singh Lakhesar et al., 2010). In Petri dishes, displacement of *Fusarium pseudograminearum* from straw by *Fusarium equiseti* and *Fusarium nygamai* was higher when urea and nitrate were used as nitrogen sources than when using ammonium as a source of nitrogen. The population of *Fusarium graminearum* on buried wheat straw was increased with ammonium nitrate, while nitrolime (calcium cyanamide) decreased the populations (Yi et al., 2002). Also the conidial production of *F. graminearum* was significantly decreased in the field by spraying urea onto pieces of maize stems (Khonga and Sutton, 1991). The antagonistic activity of *T. harzianum* was developed against the mycelial growth and the viability of sclerotia of *S. rolfii* in present of three nitrogen sources urea, ammonium sulfate and potassium nitrate (Khattabi et al., 2004). When nitrate was used as nitrogen source, the suppression of *S. rolfsii* was greater in soil by *T. harzianum* compared with urea and ammonium (Khattabi et al., 2004). *Fusarium* species used both nitrate and ammonium as a source of N, while *Trichoderma* species favorably used ammonium as a source of N (Celar, 2003).

Jabnoun-Khiareddine et al. (2016) found that potassium salts decreased tomato wilt severity incited by Fol and *V. dahliae*, and the potassium salts also enhanced growth of the tomato plant. Organic and inorganic salts had various effects on the suppression of microbial strains and the inhibition of growth of fungi in amended PDA medium was dependent on the concentration used (Fagundes et al., 2013; Olivier et al., 1998). The inhibition of growth of *Botrytis cinerea* was induced by adding calcium to the medium (Volpin and Elad, 1991).

Calcium decreased fungal infection of *Botrytis cinerea* and *Penicillium expansum* through direct inhibition of growth and germination (Wisniewski et al., 1995). The growth of *Leucostoma persoonii* was reduced 85% by using calcium propionate, 76% by using calcium hydroxide and 73% by using calcium silicate (Biggs and El-Kooli, 1994). Golden Delicious apples treated with calcium chloride solutions of 2% or 4% alone reduced decay by 40% and 60% respectively (Conway et al., 1994).

Previous studies suggested that the controlling of Fe availability in the infection court, causing Fe competition, can induce suppressiveness to Fusarium wilt pathogens (Scher and Baker, 1982). Selected isolates of *P. fluorescens* (Pf4-92 and PfRsC5) and *P. aeruginosa* (PaRsG18 and PaRsG27) produced more salicylic acid at low iron (10mM EDDHA) than high iron availability (10 mM Fe EDDHA), and all isolates produced more pseudobactin at low iron than high iron availability except PaRsG27 (Saikia et al., 2005).

The aim of this study was to determine the effect of nutrients on the antagonistic action of non-pathogenic *F. oxysporum* against the pathogen, as well as the effect of nutrients on the antibiotic production of non-pathogen on the growth of the pathogen.

6.2. Materials and Methods

6.2.1. Effect of nutrients on antagonism in dual culture

To study the effect of different nutritional elements on the antagonist action of F1 and F4 against the mycelial growth of pathogen F5769, all nutrients were tested on Czapek Dox agar medium. The recipe for the medium was varied to give different levels of the nutrients. For nitrate levels, the quantity of $NaNO_3$ in the medium was varied (0.4, 1.2, 2, 2.8, 3.6 g/l). For ammonium, NH_4Cl was substituted for the $NaNO_3$ and five concentrations of NH_4Cl were used (0.24, 0.72, 1.24, 1.76, 2.24g/l). These gave the same range of concentrations of N in the medium as for $NaNO_3$. For calcium, $Ca(NO_3)_2$ was substituted for some of the $NaNO_3$ and five concentrations were used (0, 0.96, 1.92, 2.89, 3.85g/l). The total concentration of N was kept constant. For potassium, the quantity of KCl was varied (0.32, 0.4, 0.5, 0.56, 0.64 g/l).

Mycelial plugs of 5 mm diam from the margin of 6 day old cultures of pathogen and non-pathogens were used. Both the antagonist and the pathogen were simultaneously inoculated at the opposite ends 5 cm apart from each other in the Petri dishes. In controls the pathogen was plated alone on one side of the plate at the periphery. There were 3 replicate Petri plates

for each concentration. Inoculated plates were incubated at 25 °C for 7 to 10 days. Results were expressed as means of radial fungal growth and percent inhibition of growth of the pathogen. Percent inhibition was calculated as described in chapter 3. This experiment was repeated for both $NaNO_3$ and KCl, using the highest concentration (0.56, 0.64 g/l) and lowest concentration (0.4 and 0.32 g/l) respectively (Khilare and Ahmed 2011; Ramezani, 2010; Subhani et al., 2013; Whipps, 1987).

To study the effect of interactions between N and K on the antagonist action of F1 and F4 against the mycelial growth of the pathogen, both of them were tested on Czapek Dox agar medium. The quantity of NaNO3 and KCl in the medium was used as source of N and of K respectively. High level of N and low level of K (3.6 and 0.32 g/l) were used together and lowest level of N with highest level of K (0.4 and 0.64 g/l) were used together. Plates were inoculated, incubated, and inhibition calculated as above.

6.2.2. Effect of nutrients on antibiotic production

To study the effect of different nutritional component on the antibiotic action of F1 and F4 against the mycelial growth of pathogen F5679, all fungi were grown on modified Czapek Dox liquid medium. Concentrations of $NaNO_3$, NH_4Cl, $CaNO_3$ and KCl were varied in Czapek Dox liquid medium as described above. Mycelial plugs of 5 mm diam from the margins of 6 day old cultures of F1, F4 and F5769 were transferred to each of 500 ml flasks contain 250 ml of medium and incubated at 25^{o}C on a rotary shaker at 120 rpm for 21 days. The mycelial biomasses were filtered through Miracloth and filter paper (Whitman grade 41) and then the culture filtrate was filtered through a 0.45 µm membrane. 5 ml of culture filtrate from each concentration was added to sterile Petri dishes (9 cm) and then 15 ml ¼ PDA was added and mixed carefully. A 5 mm disc from a 6 day old culture of the pathogen was used for inoculation. The inoculated Petri dishes were incubated at 25^{o}C. Each treatment had three replicates. Radial growth of the pathogen was measured. Growth on media amended with culture filtrate of the pathogen was used as a control. The inhibition due to antibiotic effect was calculated by dividing the difference in growth between the non-pathogen extract and the pathogen extract by the growth on pathogen extract (Chen et al., 2012; Khilare and Ahmed 2011; Schalchli et al., 2012; Whipps, 1987).

6.2.3. Effect of iron on antagonism in dual culture

To study the effect of different levels of iron on the antagonist action of seven antagonists against the mycelial growth of pathogen F5769, all levels were tested on Czapek Dox agar medium. To vary iron concentrations, the quantity of $FeSO_4$ in the medium was varied (0.0001, 0.001, 0.1, 0.2, 0.5 g/l). Dual culture plates were inoculated and incubated as described above, and percent inhibition of radial growth of the pathogen was calculated. Each concentration had three replicates

6.2.4. Effect of N and K on antagonism in planta

Tomato seeds of cultivar Grosse Lisse were planted in plug trays (plug size 3.4 by 3.4 by 5 cm; 6 plugs/tray), containing non sterile soilless potting mix. After 2 weeks tomato plants were transplanted into 16 cm diameter pots containing non sterile soilless potting mix. After one week conidial suspensions of non-pathogenic *F. oxysporum* (200 ml of approximately 10^6 conidia/ml) were added to 2 kg of potting mix and then after two days the conidial suspensions of pathogen F5769 (200 ml of approximately 10^6 conidia/ml) were poured into each pot..

Nutrients were prepared at three concentrations. For nitrogen levels, the quantity of NH_4Cl was used as source of N (2.1, 8.7, 17 g/10 l). For potassium, the quantity of KCl was used as source of K (0.65, 2.6, 10 g/10 l). 300ml from each concentration was watered into each pot weekly. Each treatment consisted of three replicate pots of 1 plant per pot. Disease suppression was observed by comparing growth between treatments and control (pathogen only). After 6 weeks plant height, and shoot and root dry weights were determined. Shoot height was taken from the base of the stem to apex. The shoots and roots were dried in an oven at 70^{o}C until constant weight, then weighed individually recorded (Larkin and Fravel, 2002; Scher and Baker, 1982).

6.2.5. Statistical analyses

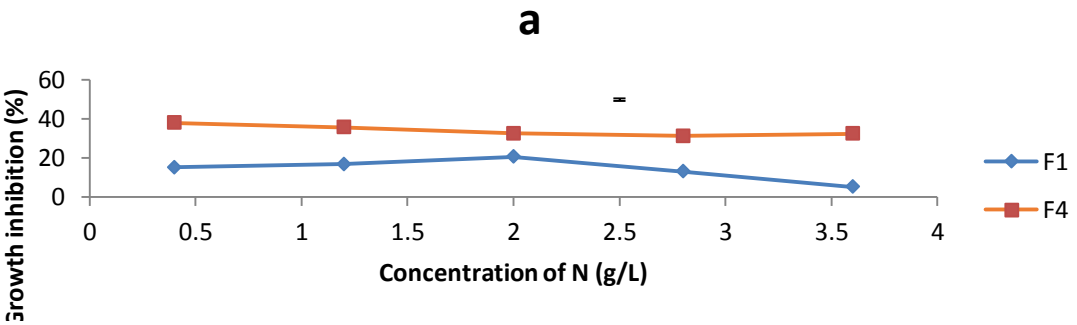
All experiments were analysed by ANOVA with a significance of $P < 0.05$.

6.3. Results

6.3.1. Effect of nutrients on antagonism in dual culture

In dual cultures of F1 or F4 with F5769 on the Czapek Dox agar medium containing different concentrations of $NaNO_3$ as source of N, F4 showed significantly stronger inhibition than F1 on mycelial growth of F5769 (Figure 6-1). The antagonistic action of F4 and F1 against pathogen F5769 increased significantly with decreasing concentration of N. As well, the effect of changes in N concentration on the inhibition was significantly stronger for F1 than it was for F4.

In the repeat experiment, the result was the same as the first experiment. There were significant differences between F1 and F4 in inhibition of mycelial growth of 5769 in the presence of $NaNO_3$ as source of N (Figure 6-1). F4 showed stronger inhibition than F1 on mycelial growth of 5769. In addition, the level of inhibition of growth of 5769 increased as N concentration decreased.



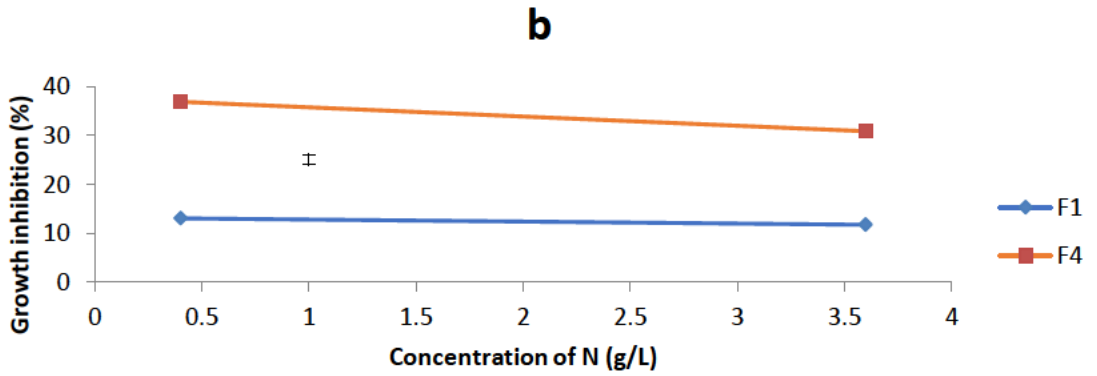


Figure 6-1 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of $NaNO_3$ as sole nitrogen source in two experiments (a and b). Error bar shows standard error for comparing any two data points (n=3).

On the Czapek Dox agar medium containing NH_4Cl as source of N, there was a significant effect of N on the inhibition of mycelial growth of pathogen 5769. For both non-pathogens F1 and F4, inhibition was greatest at high and low concentration of N and decreased at intermediate level (0.3g/l) (Figure 6-2). There was no significant difference between F1 and F4 in inhibition of F5679 when grow on NH_4Cl as N source

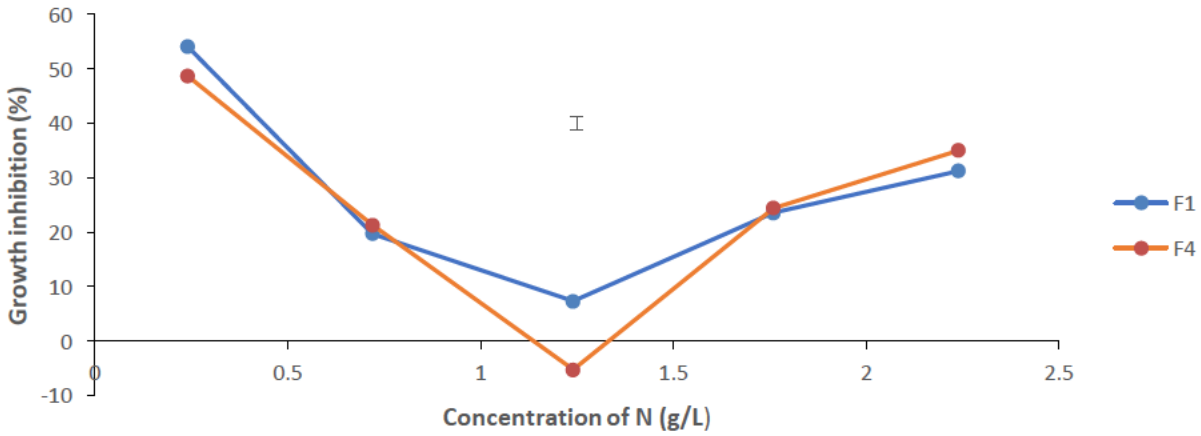


Figure 6-2 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of NH_4Cl as sole nitrogen source. Error bar shows standard error for comparing any two data points (n=3).

The potassium chloride caused significant difference between F4 and F1 on the inhibition of mycelial growth of F5769 in dual culture. The level of inhibition growth of F5769 increased as K concentration decreased (Figure 6-3). As well, F4 showed stronger inhibition than F1 on mycelial growth of 5769. The effect of K on the inhibition was stronger for F4 than it was for F1.

In the repeat experiment, the result was similar to the first experiment. There were significant differences between F1 and F4 on the inhibition of mycelial growth of 5769 (Figure 6-3). F4 showed stronger inhibition than F1 on mycelial growth of 5769. Moreover, the level of inhibition of growth of 5769 increased as K concentration decreased.

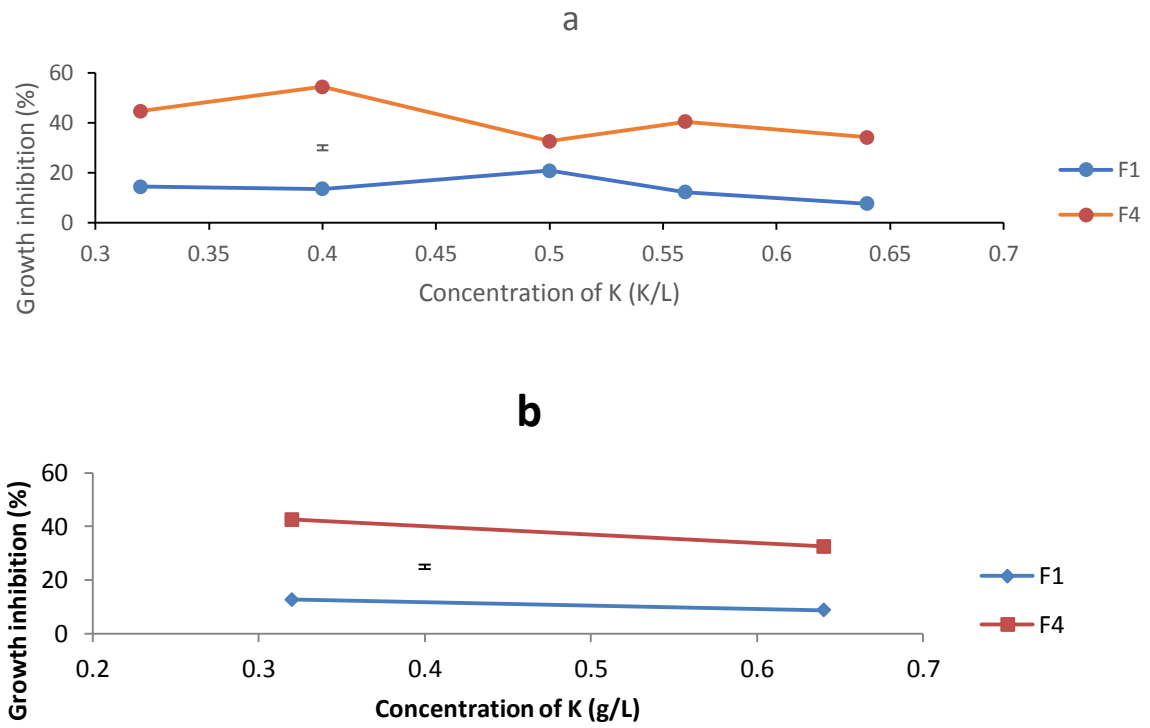


Figure 6-3 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of KCl in two experiments (a and b). Error bar shows standard error for comparing any two data points (n=3).

On Czapek Dox agar medium containing different concentrations of Ca, the inhibitory effect of F4 on the mycelial growth of the pathogen F5769 was significantly greater than F1. Also, the level of inhibition of growth of F5769 increased as Ca concentration decreased (Figure 6-4). The effect of Ca on the inhibition was stronger for F4 than it was for F1

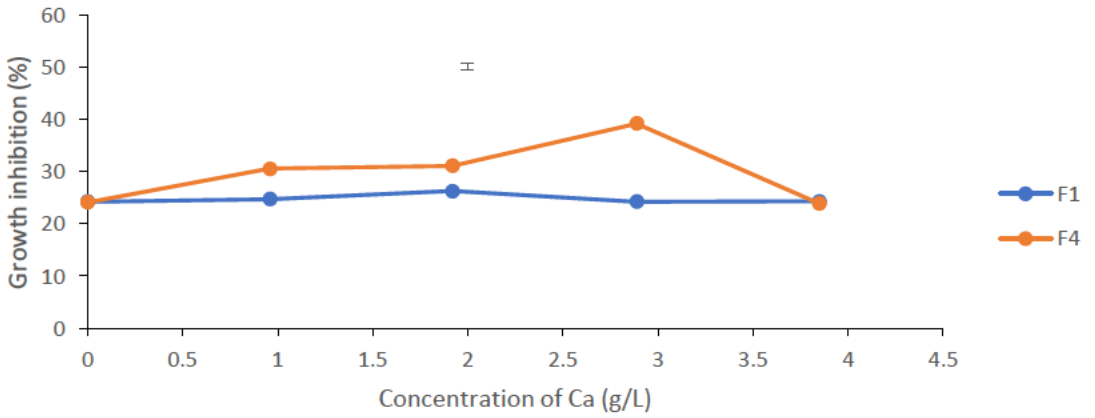


Figure 6-4 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with a range of concentrations of $CaNO_3$. Error bar shows standard error for comparing any two data points (n=3).

In dual cultures of F1 or F4 and 5769 on the Czapek Dox agar medium containing high N and low K, or low N and high K, F4 showed significantly stronger inhibition than F1 on mycelial growth of F5769 (Figure 6-5). However, the nutrient treatment did not have a significant effect on the inhibition of growth of F5769 by either F1 or F4.

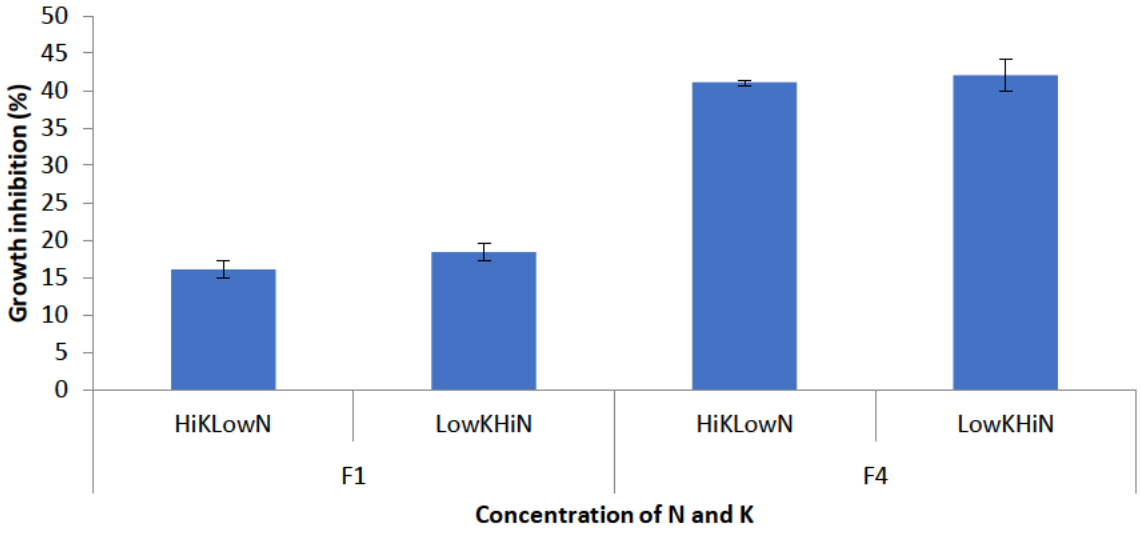


Figure 6-5 Inhibition of growth of Fol isolate F5679 by antagonists F1 and F4 on Czapek-Dox agar with highest and lowest concentration of N (3.6g/L and 0.4 g/L) and K (0.64g/L and 0.32 g/L). Error bars show standard errors (n=3).

6.3.2. Effect of iron on antagonism in dual culture

In dual cultures of non-pathogens and F5769 on the Czapek Dox agar medium, containing five levels of iron, there was a significant effect of non-pathogens on the mycelial growth of

F5769. F3 and F5 showed stronger inhibition than the other non-pathogens on mycelial growth of F5769, while F4 was recorded as having least effect on the inhibition of growth of pathogen 5769 (Figure 6-6). In addition, the level of inhibition of growth of F5769 increased as iron concentration decreased (Figure 6-7). The interaction between non-pathogen isolate and iron concentration was not significant.

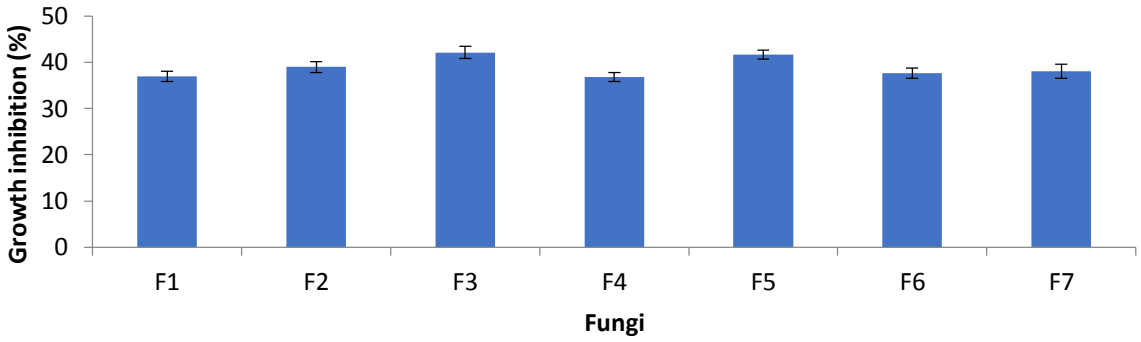


Figure 6-6 Inhibition of growth of Fol isolate F5679 by antagonists on Czapek-Dox agar with a range of concentrations of $FeSO_4$. There was no significant interaction between isolate and concentration of $FeSO_4$. Error bars show standard errors (n=3).

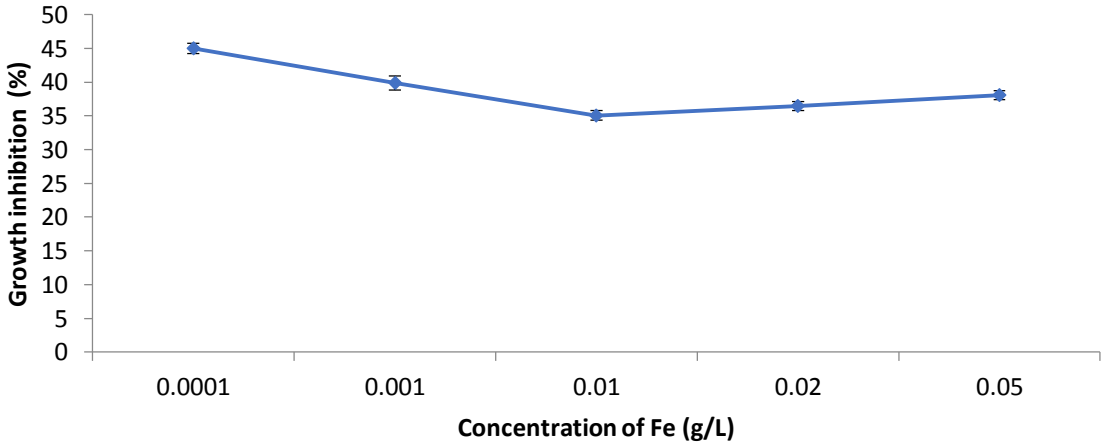


Figure 6-7 Inhibition of growth of Fol isolate F5679 on Czapek-Dox agar with a range of concentrations of $FeSO_4$. Error bars show standard errors (n=3).

6.3.3. Effect of nutrients on antibiotic production

In general, adding culture filtrates of non-pathogens produced on different levels of $NaNO_3$ to ¼ PDA medium showed that F4 had significantly stronger inhibition than F1 on mycelial growth of F5769 (Figure 6-8). The level of inhibition of growth of F5769 increased as N

concentration increased and the effect of N on the inhibition was stronger for F4 than it was for F1.

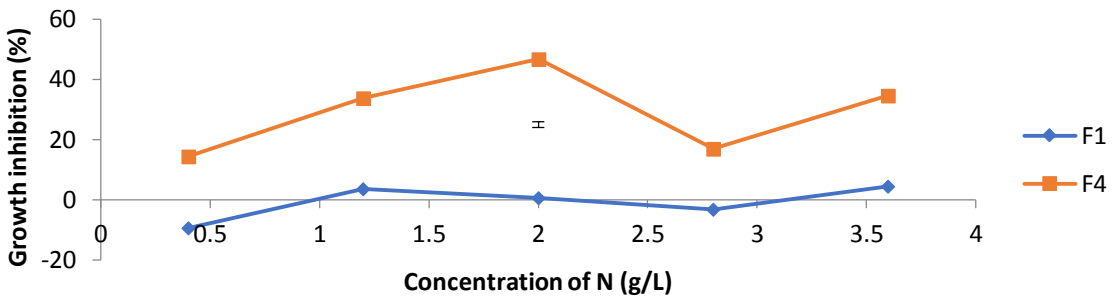


Figure 6-8 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of $NaNO_3$ as sole nitrogen source. Error bar shows standard error for comparing any two data points (n=3).

Culture filtrate of F4 from media containing NH_4Cl as source of N showed stronger inhibition than F1 on mycelial growth of F5769. For both non-pathogens, inhibition was greatest at the high and low concentrations of NH_4Cl and decreased at intermediate levels (Figure 6-9).

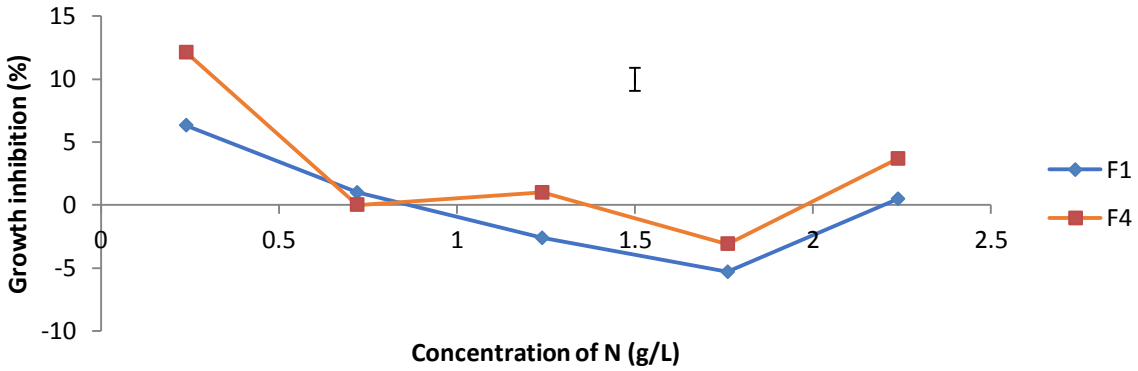


Figure 6-9 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of NH_4Cl as sole nitrogen source. Error bar shows standard error for comparing any two data points(n=3).

In general, adding filtrate of non-pathogen produced from growth on different levels of KCl to ¼ PDA medium showed significantly stronger inhibition by F4 than F1 on mycelial growth of F5769 (Figure 6-10). The level of inhibition growth of F5769 increased as KCl concentration increased and the effect of KCl on the inhibition was stronger for F4 than it was for F1.

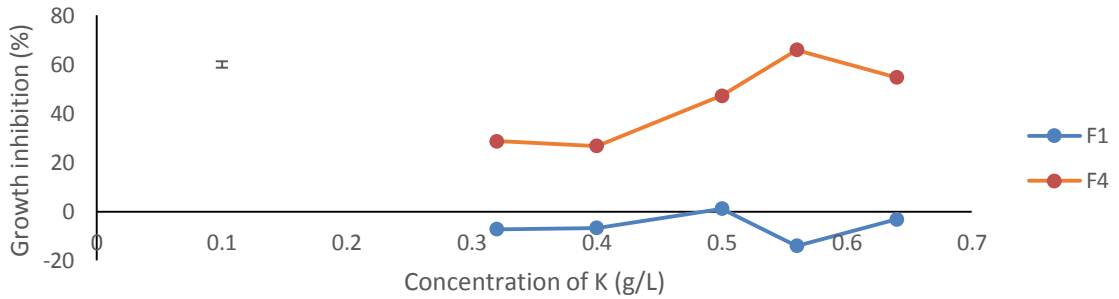


Figure 6-10 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of KCl. Error bar shows standard error for comparing any two data points(n=3).

Adding the filtrate produced from growth of non-pathogens on different levels of Ca to ¼ PDA showed stronger inhibition by F4 than F1 on mycelial growth of F5769 (Figure 6-11). The inhibition of growth of pathogen F5769 by F4 was increased as Ca decreased, while the level of inhibition of growth of F5769 by F1 increased as Ca concentration increased. The effect of Ca on the inhibition was stronger for F4 than it was for F1.

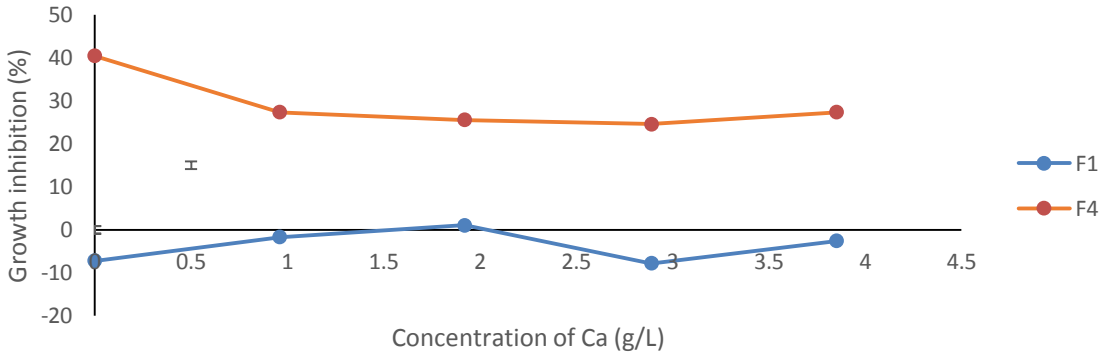


Figure 6-11 Inhibition of growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of $CaNO_3$. Error bar shows standard error for comparing any two data points (n=3).

Growth of pathogen F5769 on culture medium containing filtrate of F1 and F4 grown on different concentrations of iron was inhibited more by F4 than by F1 (Figure 6-12). The level of inhibition of growth of F5769 increased as iron concentration decreased. The effect of iron on the inhibition was stronger for F4 than it was for F1.

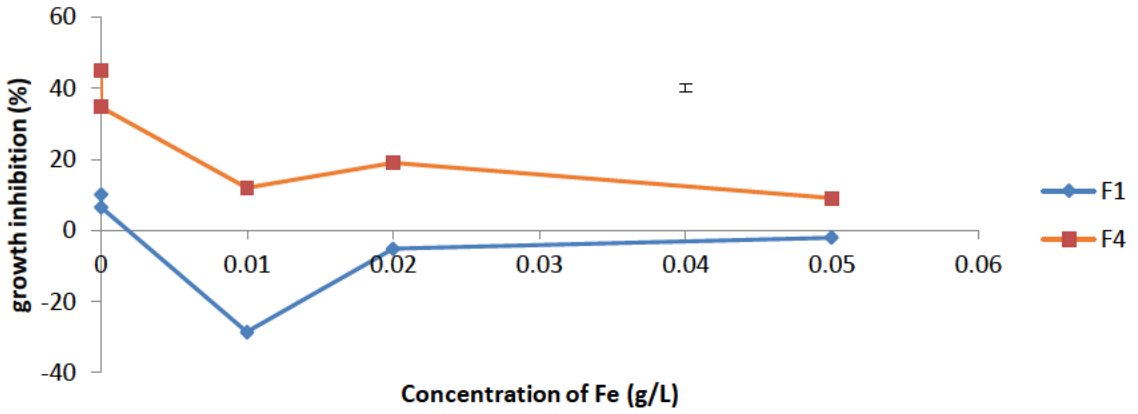


Figure 6-12 Inhibition growth of Fol isolate F5679 by filtrate of F1 and F4 on ¼ PDA with a range of concentrations of iron. Error bar shows standard error for comparing any two data points (n=3).

6.3.4. Effect of nitrogen on antagonism in planta

Tomato plant height and shoot and root dry weights were significantly higher at high concentrations of N, than at low concentration of N (Figures 6-13, 6-15). Non-pathogens (F1 and F4) significantly improved the height and shoot and root dry weight of tomato plants. However, there was a significant interaction between nitrogen level and non-pathogen. The increase in growth due to F1 and F4 was greatest at the lowest level of nitrogen, only small at the medium level of nitrogen and insignificant at the highest level of nitrogen (Figures 6-13, 6-15).

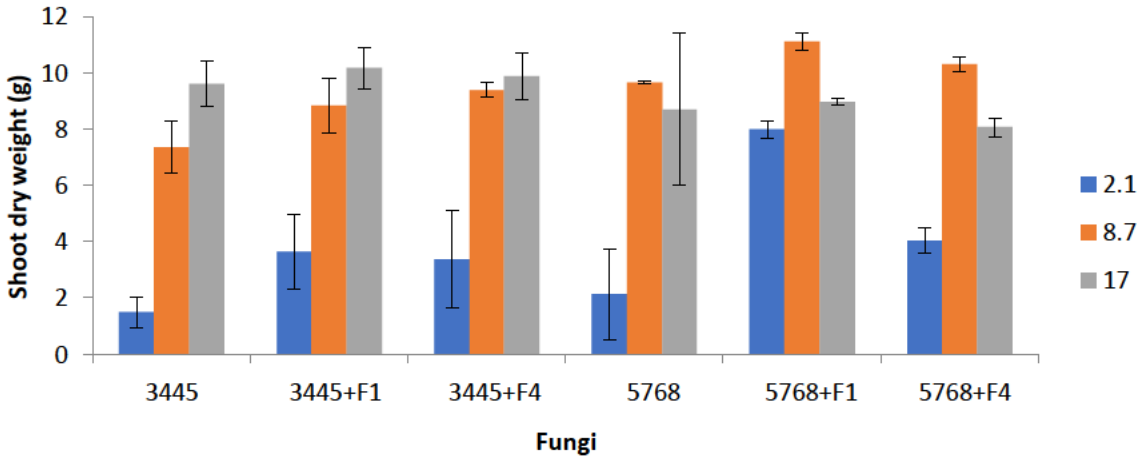


Figure 6-13 Effect of different concentrations of N (2.1, 8.7 and 17 g N/ 10L) on shoot dry weight of tomato plant when introduced into conductive soil infested with pathogens F5768, F3445 and with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=3).

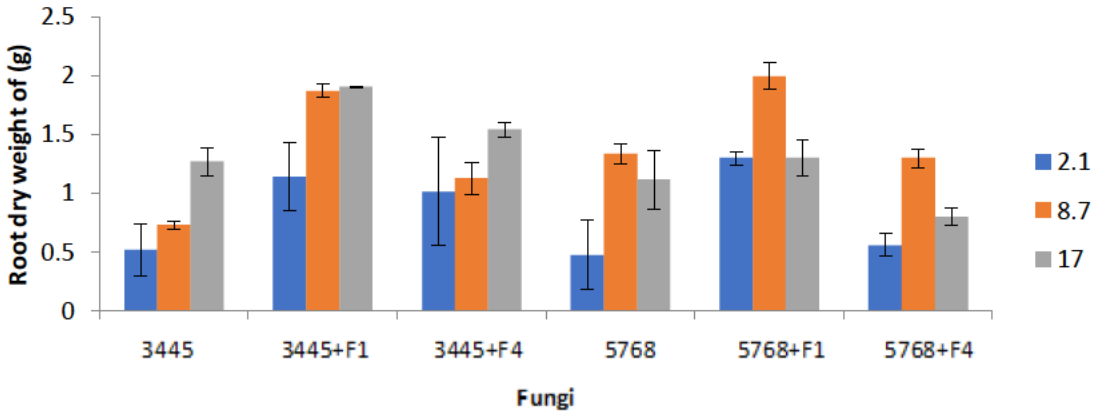


Figure 6-14 Effect of different concentrations of N (2.1, 8.7 and 17 g N/ 10L) on root dry weigh of tomato plant when introduced into conductive soil infested with pathogens F5768, F3445 and with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=3).

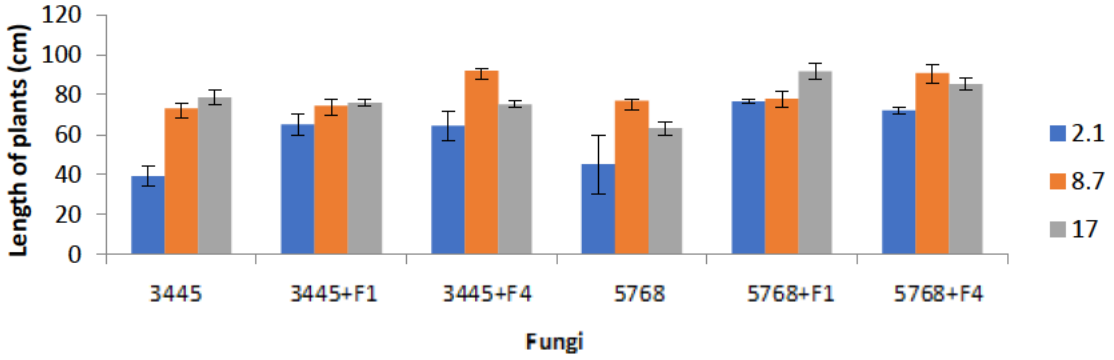


Figure 6-15 Effect of different concentrations of N (2.1, 8.7 and 17 g N/ 10L) on height of tomato plant when introduced into conductive soil infested with pathogens F5768, F3445 and with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=3).

6.3.5. Effect of potassium on antagonism in planta

The interaction between the effects of non-pathogen and potassium on shoot weight differed between the pathogens F3445 and F5768. Non-pathogens (F1 and F4) significantly increased the shoot dry weight of tomato plants in the presence of pathogen F3445 (Figure 6-16). The increase in shoot dry weight due to F1 and F4 was greatest at the lowest level of potassium, only small at the medium level of potassium and insignificant at the highest level of potassium. In the absence of non-pathogens, increasing potassium concentration increased the shoot dry weight. Shoot dry weight was high at all levels of potassium in plants treated with F5768 only. Non-pathogens (F1 and F4) had a negative effect on the shoot dry weight in the presence of pathogen F5768 at high and low concentrations of potassium. There was a slight improvement in shoot dry weight at medium level of potassium.

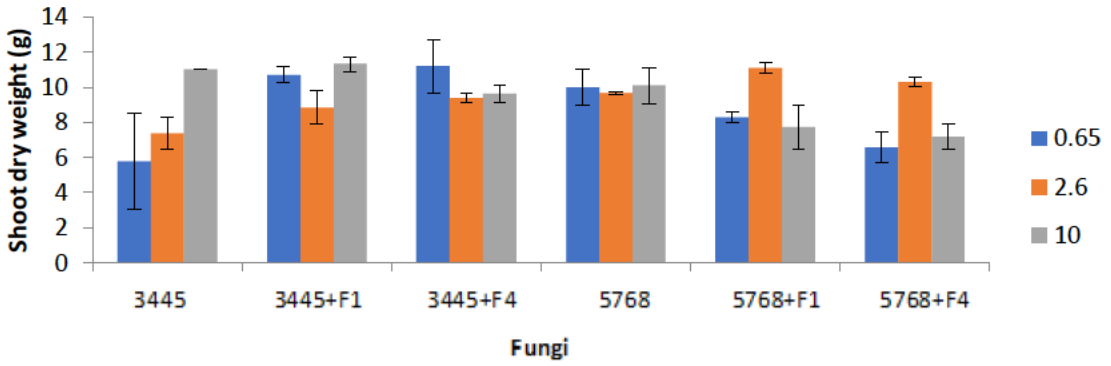


Figure 6-16 Effect of different concentrations of K (0.65, 2.6 and 10 g K/ 10L) on shoot dry weight of tomato plant when introduced into conductive soil infested with pathogens F5768, F3445 and with or without F1 and F4 (200ml of approximately 10^6 conidia/ml) Error bars show standard errors (n=3).

The effect of non-pathogen and potassium on the roots was similar to that on the shoots. However, F1 gave greater growth promotion than F4 in plants inoculated with F3445, and root weight of plants inoculated with F5768 only increased with increasing potassium concentration (Figure 6-17).

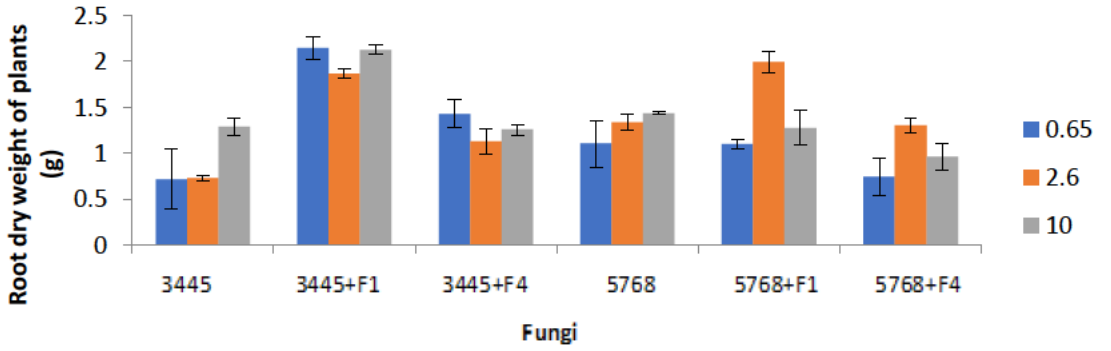


Figure 6-17 . Effect of different concentrations of K (0.65, 2.6 and 10 g K/ 10L)on root dry weigh of tomato plant when introduced into conductive soil infested with pathogens F5768, F3445 and with or without F1 and F4 (200ml of approximately 10^6 conidia/ml) Error bars show standard errors (n=3).

Non-pathogens (F1 and F4) significantly increased the height of tomato plants (Figure 6-18). The interaction between the effects of non-pathogen and potassium on height differed between the pathogens F3445 and F5768. With F3445, increasing potassium increased height in plants inoculated with the pathogen only, but not in plants treated with F1 or F4. There was no obvious relationship between potassium level and height in plants inoculated with 5768.

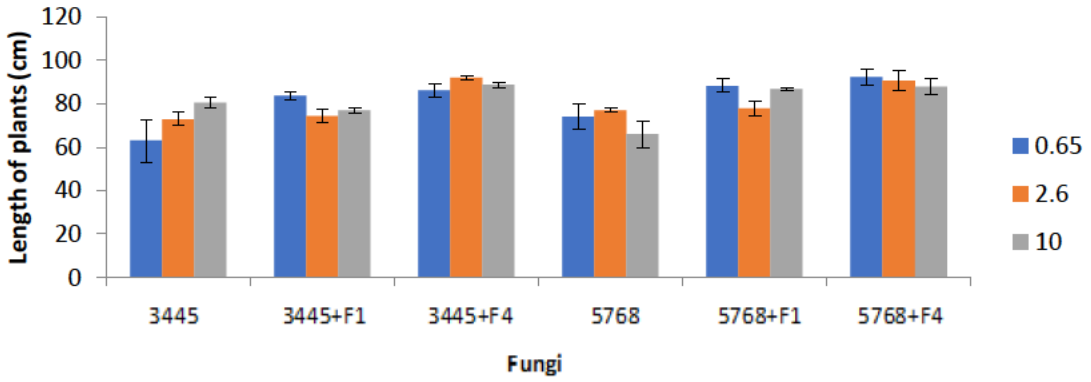


Figure 6-18 . Effect of different concentrations of K (0.65, 2.6 and 10 g K/ 10L) on height of tomato plant when introduced into conductive soil infested with pathogens F5768, F3445 and with or without F1 and F4 (200ml of approximately 10^6 conidia/ml). Error bars show standard errors (n=3).

6.4. Discussion

The dual culture trial of pathogen F5769 and non-pathogens F1 and F4 on Czapek Dox agar medium containing different sources of N revealed differences in the expression of the antagonistic action in relation to type of nitrogen source. The inhibition of F5769 by F1 and F4 decreased with increasing concentration of $NaNO_3$ as source of N. However, inhibition was greatest with both non-pathogens F1 and F4 at low and high concentration of NH_4Cl as source of N. Khattabi et al. (2004) found that each of the fertilizers ammonium sulfate, potassium nitrate and urea stimulated a different combination of mechanisms of antagonistic activity of *T. harzianum*, such as toxin production. Something similar could have occurred in this experiment.

With nitrate as sole N source, antibiotic production by F1 and F4 increased at higher N concentration, but antagonism in dual culture decreased. This result could be due to competition for N between non-pathogen and pathogen at low concentration of $NaNO_3$ as source of N. This is consistent with the results of Elad and Chet (1987) who found that the limitation in the general population of bacteria along the roots caused by the biocontrol agents is probably due to competition for nutrient.

The filtrate of F4 produced from growth on different levels of $NaNO_3$ as source of N showed stronger inhibition than F1 on mycelial growth of F5769. However, this difference was not seen when NH_4Cl was used as N source. This could be because F4 was more sensitive to the NO_3 than F1, so the secondary metabolite production of F4 was higher compared with F1. This difference could explain why F4 showed stronger inhibition than F1 on mycelial growth

of F5769. This result agreed with El-Katatny et al. (2000) who found that anti-fungal enzyme production by *T. harzianum* was significantly affected by the nitrogen source combined into the medium. The level of inhibition growth of F5769 by culture filtrate increased as N concentration increased. These results could be because N stimulated the non-pathogens to produce secondary metabolites and enzymes that could have an effect on the growth of pathogen. This is supported by Donzelli and Harman (2001); El-Katatny et al. (2000) who found that nitrogen sources such as ammonium sulfate, urea and potassium nitrate, affected the specific enzyme production by *Trichoderma*.

When NH_4Cl was used as source of N the inhibition of growth of pathogen F5769 in dual culture, and antibiotic production, was greatest at the high and low concentrations of NH_4Cl for both non-pathogens. This could be due to metabolite production of the non-pathogens being enhanced at high concentrations of NH_4Cl as sources of N. This is supported by Donzelli and Harman (2001) who found that the activities of N-acetylhexosaminidase and endochitinase were noticeably higher at 48 and 72h in a biocontrol strain of *Trichoderma*, in the presence of high ammonium and low glucose levels.

The mechanisms of antagonism by non-pathogens are complex and diversified such as competition for nutrients and induced resistance. This indicates that the effect of nitrogen form on antagonism is more complex than just an effect on growth. So, there were direct and indirect effects of nitrogen sources on the mechanism of activity of non-pathogens.

The dual culture trial of pathogen F5769 and non-pathogen F1 and F4 on the Czapek dox agar medium containing potassium chloride result showed that K had a significant effect on antagonism. The inhibition of growth of the pathogen by the non-pathogens was highest at low level of K. Low concentrations of K created competition between non-pathogen and pathogen for the nutrients, therefore the non-pathogens could grow faster than the pathogen to get nutrient. Elad and Chet (1987) and Karimi and Zamani (2013) found that when fungi grow faster, the level of chitosan production is higher which have an effect on the growth of pathogen. This result was confirmed by Safaei et al. (2016) who found that potassium can enhance chitosan production by fungi.

High concentrations of potassium chloride increased the production of antibiotics by F4. This was due to the effect of concentration of K on the secondary metabolite production by the non-pathogen. This could be due higher growth rate of F4 at higher concentrations of K.

High levels of calcium had a negative effect on antagonism by the non-pathogens against the pathogen. This could be due to the growth of both non-pathogen and pathogen being reduced by using high concentration of calcium. This result agreed with Zielinska et al. (2000) who found that *Fusarium culmorum* growth on the medium containing calcium led to inhibition of growth and development of this pathogen.

Calcium had different effects on the secondary metabolite production by non-pathogesn F1 and F4. The inhibition growth of pathogen F5769 by culture filtrate of F4 was increased at low concentration of Ca. This could be because the Ca may reduce fungal growth of non-pathogen at high concentration of Ca. This is supported by Wisniewski et al. (1995) who found that the processes of growth and spore germination of *Botrytis* were affected by using Ca They also found that isolate 247 of *Candida oleophila* did not inhibit the pathogen when $CaCl_2$ was used. However, the inhibition of growth of pathogen F5769 by culture filtrate of F1 was increased at high concentration of Ca. This result could be because F1 may not be affected by toxic levels of Ca while the pathogen was affected. This result agreed with Wisniewski et al. (1995) who found that the pathogens *Botrytis cinerea* and *Penicillium expansum* were inhibited by increasing concentration of calcium, whereas the growth of the yeast isolate 182 of *Candida oleophila* was not affected.

Dual cultures of non-pathogens and F5769 on Czapeck-Dox agar medium containing five levels of Iron, showed the level of inhibition of growth of F5769 increased as iron concentration decreased. This result could be because at high level of iron there was enough Fe available for both fungal pathogen and non-pathogen, whereas with low level of iron there was not enough available iron for both fungi, so that could create competition between the pathogen and non-pathogen which led to reduce growth of the pathogen. This result is in agreement with Simeoni et al. (1987) who found that when Fe concentration dropped to between 10^{-22} and 10^{-27} M, Fe competition of *Pseudomonas putida* was enhanced and germination of *Fusarium oxysporum* f.sp. *cucumerinum* was significantly decreased.

Iron had different effects on the secondary metabolite production of F1 and F4. Culture filtrate of F4 showed stronger inhibition than F1 on mycelial growth of F5769. This result could be because F1 was more sensitive to the iron level than F4. Concentration of iron had a big influence on the growth of F1 compared with F4, so the biomass of F4 was bigger than F1 which led to greater production of secondary metabolites that had influence on the growth of pathogen. Also the inhibition of growth of the pathogen at low concentration of iron was

greater compared with high concentration of iron. This could due to a high concentration of iron causing stress to the non-pathogen F1 and F4. So the growth of both non-pathogen were less compared with low concentration. Therefore the amount of secondary metabolites which affected the growth of the pathogen was highest at low concentration compared with high concentration of iron. This result agrees with that of Leeman et al. (1996) who found that at low iron availability in vitro, *P. fluorescens* WCS374 and WCS417 produced 47 and 8 mg of salicylic acid per millilitre, respectively, whereas the SA production by the both strains was decreased with increasing iron availability.

In glasshouse experiments, the growth of shoot, root and height of tomato plant at high concentration of nitrogen, was greater than at low concentration of nitrogen. This is because nitrogen plays an important role in growth of tomato plants. The effect of the non-pathogens in antagonism of the disease was greatest at low levels of nitrogen fertilization. The inhibition of virulence of pathogen at low concentration of nitrogen could be due to first, nitrogen had negative aeffect on the growth of pathogen at low concentration. This result agrees with Sharma et al. (2013) who found that low levels of nitrogen in soil beneath *Azadirachta indica* inhibited growth of *F. oxysporum*. Second, at low concentration of N there was competition between non-pathogen and pathogen for the nutrient. So the non-pathogen could establish on the root surface and prevent the pathogen from infecting the plant. This agrees with Elad and Chet (1987) who found that the limitation in the general population of bacteria along the roots caused by the biocontrol agents is probably due to competition on the nutrient. Also, the form of nitrogen which is added to soil could have a different effect on the growth of fungi. So, adding ammonium to the soil as a source of nitrogen could increase growth of the pathogen. This agrees with Lakhesar et al. (2010) who found that adding ammonium to the medium increased the radial growth of *Fusarium pseudograminearum* whereas, radial growth of *F. pseudograminearum* was decreased by adding nitrate. Also F1 and F4 had less effect at high concentration of N is because the plant is more tolerant to Fol when it has more N. This results agreed with Sarhan et al. (1982) found that disease incidence of Fol decreased with high levels of N.

In glasshouse experiments, at low concentration of K, F1 and F4 increased shoot dry weight and that could be because potassium had a negative influence on the growth of non-pathogen at high concentration of K. So the role of F1 and F4 to improve health of tomato plant found in previous experiments become less. This result agreed with Jabnoun-Khiareddine et al.

(2016) who found that potassium salts had different effects on the inhibition of fungal growth and reduced disease severity of tomato plant by Fol. Also the result showed increasing potassium concentration to soil improved growth of plant in absence of non-pathogen. This could due to the role of potassium to develop growth of tomato plant. Jabnoun-Khiareddine et al. (2016) found that potassium salts enhanced growth of height, and root and shoot fresh weight of tomato plant.

So, nitrogen and potassium at high and low concentration have effects on growth of the fungi in dual culture, however nitrogen and potassium did not show effects on growth and antagonism between non-pathogens F1 and F4 and pathogen F5769 in glasshouse.

Chapter 7. General Discussion

The aim of the work described in this thesis was to improve the biocontrol of Fusarium wilt in tomato by understanding the environmental and host factors which effect the antagonism of the pathogen *Fusarium oxysporum* f. sp. *lycopersici* by non-pathogenic strains of *F. oxysporum*. This was done by first obtaining biocontrol agent strains of *F. oxysporum* that could be used against Fusarium wilt caused by Fol. The ability of non-pathogens to induce systemic resistance against Fusarium wilt was tested and role of iron in induced resistance was examined. Also, the effects of mineral nutrients and root exudate on antagonism between non-pathogens and pathogen were examined.

The pathogenicity of Fol was tested in two different ways, root dipping and adding conidial suspension to soil. It was found that in root dipping, the pathogens were more aggressive against tomato plants compared with adding conidial suspension to the soil. This could be because in root dipping, spores were more likely to contact the root surface and reach the elongation zone and develop disease than by adding conidial suspension (Olivain et al., 2006). So the method of adding conidial suspension to the soil was used in the next experiments because this method gave less severe disease, allowing the detection of the effects of antagonists. Also adding spores to soil is more similar to the way that infection occurs in the field. In addition, measuring the effect of disease on growth had advantages over scoring incidence or severity of wilting. The symptoms were more consistent between replicates and gave a quantitative measurement that was better suited to statistical analysis.

In dual culture and pot trials it was found that all non-pathogens reduced growth and disease severity of pathogen Fol. However, the highest growth inhibition of the pathogen was recorded by isolate F4. Isolates F1 and F4 protected all the plants from infection by the pathogen when rated by showing no brown colour in the stem. This may be because F1 and F4 have the greatest competition for nutrients or antibiotic production compared with the other non-pathogens (Fravel et al., 2003; Kumar and Garampalli, 2013; Postma and Luttikholt, 1996). Also, F1 and F4 improved the growth of tomato plants infected with Fol. Therefore, F1 and F4 were chosen for further experiments.

Isolates F1 and F4 reduced the growth of tomato plants when applied in the absence of Fol inoculation. A previous study found that nonpathogenic biocontrol *F. oxysporum* strain 70T01 had the ability to colonize the cortex of tomato roots and the densities in roots were

highest at inoculation sites compared with away from the inoculation sites (Bao and Lazarovits, 2001). However, there appear to be no reports on the effects of root colonization by biocontrol strains on tomato growth. The antagonists are called 'non-pathogens' because they do not cause wilting or vascular browning, but they could still be parasites that reduce the plant growth. So non-pathogens should not generally be used in the absence of disease.

F1 and F4 reduced the percentage of germination of tomato seeds, so the methods of using non-pathogen F1 and F4 for the next experiments were to apply them after germination of the seeds. It could be that antibiotic production by F1 and F4 had an effect on the seed germination (Ibraheem et al., 1987; Owolade et al., 2005). So non-pathogens should be added to seedlings before transplanting to the field.

Non-pathogens protected plants from Fusarium wilt even when there was no direct contact with the pathogen. So F1 and F4 showed ability to induce resistance in tomato plants and this could be due to either improved health of the plant or inducing specific defence mechanisms in the plant (Tamietti et al., 1993). This is consistent with earlier work on non-pathogenic *F. oxysporum* on tomatoes. Fuchs et al. (1997) found that non-pathogen Fo47 increased the chitinase, β-1, 3-glucanase, and β-1, 4-glucosidase activity in tomato plants. Also a previous study reported that adding non-pathogen *Fusarium* strains to soil led to stimulation of systemic resistance by changes in the stems and leaves of tomato plants (Tamietti et al., 1993).

Root exudates played an important role to stimulate germination of pathogen and non-pathogen spores. This could be because components of root exudates such as sugars, organic and amino acids stimulated spore germination (Nelson, 1991). However, in the presence of root exudates, all antagonists inhibited the growth of pathogen F5769 in vitro. This result is consistent with that of Buxton (1960) who found that root exudate of pea increased the ability of rhizosphere fungi to inhibit growth of the pathogen *F. oxysporum* f.sp. *pisi*.

When organic acids and sugar were used as source of carbon, both of them enhanced antifungal activity of the non-pathogens against the pathogen. Also, sugar and organic acids stimulated the non-pathogens to produce antibiotics which acted against the pathogen (Kravchenko et al., 2003).

In dual culture, the antagonism between non-pathogens F1 and F4 and pathogen F5769 was affected by different sources of N. With increasing concentration of $NaNO_3$ as source of N,

the inhibition of growth of pathogen F5769 by non-pathogens F1 and F4 was decreased. However, at high and low concentration of NH_4Cl as source of N, the inhibition of growth of pathogen F5769 by non-pathogens F1 and F4 was increased. This difference in result could be due to the form of N which played an important role to stimulate or limit the mechanisms of non-pathogens which have an effect on their antagonistic activity (Khattabi et al., 2004).

At high concentration of $NaNO_3$ as sole N source, the antibiotic production of F1 and F4 was increased, whereas in dual culture the antagonism was decreased. This could be because competition for nutrient between non-pathogen and pathogen at low concentration of $NaNO_3$ as source of N (Elad and Chet, 1987) was more important than antibiotic production as a mechanism of antagonism. With different levels of $NaNO_3$ as source of N, the antibiotic production of F4 gave stronger inhibition than F1 on growth of pathogen F5769. However the levels of NH_4Cl did not have different effects on antibiotic production of non-pathogens F1 and F4. This could be because F4 was more sensitive than F1 to $NaNO_3$ as a source of N (El-Katatny et al., 2000).

Using NH_4Cl as source of N in dual culture and antibiotic production at high and low concentration led to improved inhibition of growth of pathogen F5769 by non-pathogens F1 and F4. This could be due to the antibiotic production being improved at high levels of NH_4Cl as source of N (Donzelli and Harman, 2001).

In the glasshouse, N played an important role to develop healthy plants at high levels of application. However, at low levels of N, the aggressiveness of the pathogen was reduced compared with high N. This could be because at low level of N the pathogen did not grow very well Sharma et al. (2013), or when N level was low that created competition for nutrients between non-pathogens and pathogen. Therefore it could be that the non-pathogens had the ability to establish on the root surface of plant which influenced the virulence of the pathogen (Elad and Chet, 1987). This result was consistent with what was found in the dual culture and antibiotic production experiments except with high level of N. That could be due to tomato plants with high level of N becoming more tolerant to the pathogen Fol than at low level of N. Sarhan et al. (1982) found that at high level of N the disease severity of Fusarium wilt was decreased. So the non-pathogens will be less effective when soil N levels are high. Meanwhile the non-pathogens will be more effective in soil that is poor in N.

In dual culture, the antagonistic activity of non-pathogens F1 and F4 against pathogen F5769 was enhanced at low level of K. This could be because the low level of K made the non-pathogens grow faster than the pathogen to get nutrient (Elad and Chet, 1987; Karimi and Zamani, 2013).

The antibiotic production of F4 was increased at high level of K. This could be because the growth of non-pathogen was greater in media with high level of K which could affect the secondary metabolite production.

In the glasshouse, non-pathogens improved shoot dry weight of diseased tomato plants at low level of K more than at high levels. This could be because K at high level had a negative effect on the growth of non-pathogens. However, at high level of K, the health of infected tomato plants was improved even in the absence of non-pathogens. This could be due to the role of K at high level to develop healthy plants (Jabnoun-Khiareddine et al., 2016). This result supported what was found in dual culture, where low level of K improved the antagonistic activity of non-pathogens F1 and F4 which may reduce the aggressiveness of the pathogen. So the non-pathogens will be less effective in soil rich with K. Meanwhile, the non-pathogens will have their greatest effect in soil poor in K.

The antagonistic activity of non-pathogens F1 and F4 against pathogen F5769 was negatively affected by high levels of Ca. This could be because high level of Ca affected the growth of non-pathogens (Zielinska et al., 2000), or reduced competition between the pathogen and non-pathogens for Ca.

At low level of Ca, the antibiotic production of F4 inhibited growth of the pathogen F5769. This could be due to the growth of non-pathogen being decreased at high level of Ca (Wisniewski et al., 1995). However, at high level of Ca, the antibiotic production of F1 inhibited growth of pathogen F5769. This result could be due to toxicity of Ca to the pathogen F5769 and F1 was not affected by high level of Ca (Wisniewski et al., 1995).

Iron also had various effects on the antibiotic production by non-pathogens F1 and F4. The inhibition of growth of pathogen F5769 by culture filtrate of F4 was greater than culture filtrate of F1. This could be because F1 was more influenced by iron levels than F4, so the biomass of F4 was much bigger than biomass of F1. As a result, F4 produced a larger amount of antibiotic production compared with F1 which had an effect on growth of the pathogen. Also, at low level of iron, the inhibition of growth of pathogen F5769 by antibiotic

production of non-pathogens F1 and F4 was greater compared with high level. This could be due to non-pathogens at high level of iron being exposed to stress which led to less growth. So non-pathogens F1 and F4 produced a greater amount of antibiotic at low iron level compared with high level which had influenced the growth of pathogen F5769 (Leeman et al., 1996).

The induction of resistance in tomato by non-pathogens F1 and F4was greatest when iron was at standard and high availability, this could be because at low iron concentration the pathogen could not grow very well, or the pathogen had less effect on the plant at low iron concentration. So the pathogenicity could be less (Leeman et al., 1996). On the other hand, non-pathogens may produce antibiotics with high concentrations of iron which induced systemic resistance of the tomato plant. This result contrasted with Saikia, et al. (2005) who found that at low level of iron, the *Pseudomonas* spp. caused production of more salicylic acid (SA) in chickpea than at high level of iron, which played an important role to induce resistance.

When the pathogen and non-pathogen were applied with high concentrations of iron together in solution culture, the greatest relative effect of F4 on disease occurred at low iron concentration. This could be due to competition between the non-pathogen and pathogen for iron availability before infection by the pathogen. This result is consistent with that of Scher and Baker (1982) who found that reducing the iron availability in soil can induce suppressiveness to Fusarium wilt by competition for iron in the infection site. Segarra et al. (2010) also showed that competition for iron was a key mode of action for the antagonism of Fusarium wilt in tomato by *Trichoderma asperellum*. At high levels of iron, the inhibition of growth of pathogen F5769 by non-pathogens F1 and F4 was decreased. This could be due to the availability of iron being enough for both pathogen and non-pathogen. However, at low levels of iron there was not enough iron, so that could create competition between the pathogen and non-pathogen which led to reduced growth of the pathogen (Simeoni et al., 1987).

7.1. Suggestions to improve antagonism by non-pathogens

Non-pathogens should be added to soil after seed germination to prevent decreased germination. The non-pathogen should not be applied to soil in the absence of the pathogen

as non-pathogens had a negative effect on the growth of plants when used alone. Also, adding non-pathogens to soil at least two days before transplanting seedlings to the field would allow the non-pathogens to establish on the root surface of plants and stimulate induced resistance. Seeds should be grown on sterilized soil and non-pathogen added after seed germination before transplant to the field because root exudate played an important role in stimulating spore germination for both non-pathogen and pathogen. So that gives more time for the non-pathogen to establish on the root surface.

Applying non-pathogen to soil when iron level is high should be avoided, or we should add low concentrations of iron to soil as fertilizer. Non-pathogens should not be applied to soil when N levels are high. However, we should add non-pathogens to soil poor in N. Using low concentration of N as fertilizer could improve the antagonistic activity of non-pathogens. Also care should be taken about the form of N which is added to soil as fertilizer as each form used as a source of N has a different effect on the plant and pathogen. Adding low concentration of K as fertilizer could improve the antagonistic activity of non-pathogens. Adding low concentration of Ca as fertilizer could improve the antagonistic activity of non-pathogens.

7.1.1. Suggestions for future work

It would be a good idea to study why non-pathogens had a negative effect on plant growth in the absence of the pathogen. Future work could determine if this is this because of the reaction of the host plant toward non-pathogens or because the density of non-pathogen on the root surface or root tissue can be high, so that non-pathogens could consume nutrients from the roots. An analysis of the antibiotic production of non-pathogens could be done to see if application to the plant has the ability to induce resistance of plants. That could give a good opportunity to avoid use of non-pathogens directly which sometimes when applied alone had an effect on the growth of plants. It is also important to examine the interaction between plants and non-pathogenic *F. oxysporum* because there has not been much study about whether the non-pathogens could become pathogenic or not in the future. It may also be possible to select plants for resistance to the non-pathogens so that their growth is not affected by the biocontrol agents in the absence of the pathogen.

It would be useful to find a way to apply non-pathogens with seeds without affecting seed germination, like coating the seed with some chemical to inhibit the non-pathogen from

reaching the seed until germination. Or non-pathogens could be applied with some distance from seeds, so it gives time for the seed to germinate before the fungus comes into contact with it. This is very important as it allows the non-pathogen to establish around the seed area to prevent the pathogen reaching the seed and infecting the plant. Also, it may be possible to find some materials to apply with the non-pathogen that have ability to attract the non-pathogen for a short time, so it gives time for germination of seeds. Also, more studies could be done to find the optimum concentration of non-pathogen inoculation that has no effect on the seed germination but still protects against Fusarium wilt.

It may be possible to apply sugar and organic acid to the field soil which has non-pathogens and pathogen before transplanting the seedling to the field. This may stimulate germination and interactions between pathogens and non-pathogens before sowing the plant. The non-pathogens reduced growth of the pathogen in the presence of sugar and organic acid, so the density of pathogen will decrease, and the possibility to infect the plant will be less. Experiments should test whether stimulating the plant to increase production of sugar and organic acids enhances biocontrol, because these components played an important role to develop antifungal activities of non-pathogens

High levels of iron reduced the effect of the non- pathogens, so we should find a way that can reduce iron concentration such as using siderophore-producing bacteria. That could help the use of non-pathogens in soil rich in iron without affecting the antagonism of non-pathogens against the pathogen. Also more studies should be done about the effect of iron levels on the non-pathogens by using genetic tools to develop the ability of non-pathogens to tolerate the high level of iron and retain the same ability to inhibit growth of the pathogen.

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