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Importance of the mutualistic endophyte *Fusarium oxysporum* 162 for enhancement of tomato transplants and the biological control of the root-knot nematode *Meloidogyne incognita*, with particular reference to mode-of-action

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This work is dedicated to my wife and my parents

Abstract

Importance of the mutualistic endophyte *Fusarium oxysporum* 162 for enhancement of tomato transplants and the biological control of the root-knot nematode *Meloidogyne incognita*, with particular reference to mode-of-action

A mutualistic endophyte *Fusarium oxysporum* strain 162 and four *Trichoderma* isolates were studied for bio-enhancement of tomato against *M. incognita* and their mode of action.

In vivo experiments were carried out to study the effect of the different biocontrol agents on gall and egg mass numbers of *M. incognita*. The fungi were added to the soil at two different times: at transplanting and one week before transplanting. Treatment of the soil at transplanting with fungi and *M. incognita* resulted in a reduction of up to 40 % in nematode galling. However, treating the soil with FO162 or *Trichoderma* and *M. incognita* one week before transplanting tomato seedlings resulted in gall reductions between 30.8 and 44.5% respectively.

However, in both experiments, the FO162 standard gave higher level of control when compared to all *Trichoderma* isolates. The suppressive effect of the fungi on egg mass production, which is a measure of nematode development over time, was confirmed only for FO162. *Trichoderma* isolates did not reduce egg mass number. The lack of nematode control with *Trichoderma* was probably due to the short exposure time of the nematode to *Trichoderma*.

In vitro experiments were conducted to test the effects of the different biocontrol agents on nematode mobility and mortality. Nematode immobility ranged from 10 to 28% among the biocontrol agents compared with 7.8 % and 1.3% in the media and water controls respectively. No direct parasitism on *M. incognita* was observed. Pathogenicity of the biocontrol agents toward *M. incognita* was studied in the soil without plants. Nematode mortality as affected by the different biocontrol agents ranged from 36-82 % compared to the control. FO162 when compared with the *Trichoderma* isolates gave superior nematode control of up to 82%.

The influence of FO162 application time was studied. Treatment at sowing alone as well as dual inoculation at sowing and at transplanting led to similar significant reductions in nematode damage. Multiple inoculations gave a slightly higher reduction in the number of egg masses and galls than the single treatment at sowing.

The effect of two types of inoculum levels of FO162: at sowing with 10^5 cfu/g seedling substrate and at transplanting with 10^4 cfu/g soil gave significantly higher levels of control when compared to the control. However, there were no significant differences between the doses used in the combination treatments. Inoculation of the fungus did not affect female growth nor fecundity in synchronized tests with nematodes of the same age.

The mutualistic endophyte *F. oxysporum* 162 showed different modes of action: 1) repellent action and 2) induced systemic resistance

F. oxysporum 162 colonized all *F. oxysporum* wilt resistant and susceptible tomato varieties as observed 3 weeks after fungal inoculation. The level of colonization was found to range between 40–100% among the different varieties. The reduction in nematode penetration was closely related to the percent colonization of the root with the non-pathogenic *F. oxysporum*. Higher percent colonization resulted in lower nematode penetration of up to 82%.

Zusammenfassung

Bedeutung des mutualistischen Endophytens, *Fusarium oxysporum* 162, für die Stärkung von Tomatenpflanzen und der biologischen Kontrolle des Wurzelgallennematoden, *Meloidogyne incognita*, unter besonderer Berücksichtigung der Wirkungsweise

Der mutualistische Endophyt *Fusarium oxysporum* Stamm 162, sowie vier Isolate von *Trichoderma* spp. wurden bezüglich Wirkungsmechanismen der biologischen Stärkung an Tomatenpflanzen gegenüber *M. incognita* untersucht.

In vivo Versuche wurden durchgeführt, um die Effekte der verschiedenen Pilze auf Gallenanzahl und Eiermasse von *M. incognita* zu untersuchen. Die Pilze wurden an zwei verschiedenen Terminen dem Boden zugesetzt: eine Woche vor und zum Zeitpunkt der Pflanzung der Tomaten. Wurden Pilze und Nematoden während der Tomatenpflanzung ausgebracht, so führte dies zu einer Gallenreduktion an den Tomatenwurzeln von bis zu 40%. Eine Bodenbehandlung mit Fo162 oder *Trichoderma* und *M. incognita* eine Woche vor Auspflanzung der Setzlinge führte zu Gallenreduktionen von 30,8 bzw. 44,5%.In beiden Experimenten übertrafen die Kontrollergebnisse von Fo162 die Isolate von *Trichoderma* spp. Ein suppressiver Effekt auf die Ausbildung von Eiermassen von *Meloidogyne* spp. welcher als Maß für die Nematodenentwicklung gilt, konnte nur für Fo162 bestätigt werden. Die Isolate von *Trichoderma* riefen keine Reduktion der Eiermassen hervor. Dies lag wahrscheinlich an der kurzen Expositionszeit der Nematoden gegenüber den getesteten Organismen dadurch blieb ein Kontrolleffekt aus.

In vitro Versuche wurden durchgeführt, um den Einfluss der Biokontrollorganismen auf die Mobilität und Mortalität von *M. incognita* zu bestimmen. Der Mobilitätsverlust der Nematoden lag zwischen 10 und 28% bei den Biokontrollorganismen im Vergleich zu 7,8% in einem Kontrollmedium und 1,3% in Wasser. Es wurde keine direkte Parasitisierung an *M. incognita* beobachtet. Die Pathogenität der Biokontrollorganismen gegenüber *M. incognita* wurde im Boden ohne Tomaten getestet. Die durch die Biokontrollorganismen hervorgerufene Nematodenmortalität schwankte hierbei zwischen 36 und 82% im Vergleich zur Kontrolle. Fo162 erreichte eine um bis zu 82% bessere Nematodenkontrolle als die getesteten Isolate von *Trichoderma*.

Der Einfluss des Applikationszeitpunktes wurde untersucht. Eine einmalige Behandlung zur Aussaat und eine zweifache Behandlung, zur Aussaat und zur Pflanzung, führten zu ähnlichen, signifikanten Ergebnissen bei der Reduzierung der durch Nematoden bedingten Schäden. Die mehrfache Inokulation verstärkte die Reduktion der Eiermassen und Wurzelgallen nur marginal im Vergleich zur einmaligen Inokulation zum Zeitpunkt der Aussaat.

Zwei verschiedenen Inokulationsdichten und Zeitplan wurden in verschiedene Kombination getestet. Die beste Kontrollwirkung gegenüber *M. incognita* ergab eine kombinierte Inokulation mit FO162 mit 10^5 cfu/g Boden bei der Aussaat und 10^4 cfu/g Boden bei der Pflanzung. Diese Behandlung unterschied sich signifikant von der Kontrollvariante und einigen anderen Inokulationssystemen unterschied. Die kombinierten Behandlungen zeigten keine signifikanten Unterschiede zu ein andere. Zweifache Pilzinokulation beeinflusste weder das Wachstum noch die Fruchtbarkeit der Nematodenweibchen.

Der mutualistische Endophyt FO162 zeigte folgende Wirkungsmechanismen: 1)Abwehr, und 2) induzierte Resistenz.
Drei Wochen nach der Inokulation besiedelte *F. oxysporum* 162 alle getesteten Tomatensorten, unabhängig von ihrem Resistenzgrad gegenüber Fusariumwelke. Die Stärke der endophytischen Wurzelbesiedlung betrug zwischen 40-100% für die verschiedenen Sorten. Der Besiedlungsgrad steht in engem Verhältnis zur Wirksamkeit der Nematodenkontrolle. Die Reduktion der Wurzelpenetration korrelierte stark mit der prozentualen Besiedlungsdichte durch apathogenes *Fusarium oxysporum*. Höhere Kolonisierungsraten führten zu einer Verminderung der Penetrationshäufigkeit des Wurzelsystems um bis zu 82%.

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Chapter one

GENERAL INTRODUCTION

1. The importance of tomato

Tomato (*Lycopersicon esculentum* Mill.) is a major vegetable crop that has achieved tremendous popularity over the last century. It is grown in practically every country of the world in outdoor fields, greenhouses and in net houses (Wener, 2000). World production of tomato, which ranks as a leading fresh and processed vegetable crop, exceeded 108.5 million metric tons in 2002 and occupied approximately 4.0 million hectares. In table 1.1, the overall level of production of a number of vegetable crops in the four tropical regions of the world is tabulated. World tomato production is valued at 5-6 billion US dollars with international trade amounting to 3-3.5 billion US dollars annually (FAO, 2003).

The tomato plant is a very versatile crop that can be divided into two categories by use; fresh market tomatoes, which are sold for human consumption and processing tomatoes, which are grown only outdoors for the canning industry and are mechanically harvested. In both cases, world production and consumption has grown quite rapidly over the past 25 years (Wener, 2000). Tomato, aside from being tasty, are very healthy as they are a good source of vitamins A and C (Wener, 2000; http://www.ars-grin.gov/npgs/cgcreports/ tomatocgcreport 2003.html).

Increased production is associated with major advances in production and processing technology. In addition, modern breeding methods supported by new molecular techniques are making major strides to shorten the development time for cultivars with plant resistance to nematodes, insects and diseases.

Table 1.1 Area in 1000 ha, yield in metric tonnes and total production in 1000 metric tonnes for select vegetables in regions with large tropical and subtropical climates. [a]

	Africa			C. America			S. America			Asia		
Vegetable	Area	Yield	Prod.	Area	Yield	Prod.	Area	Yield	Prod.	Area	Yield	Prod.
Cabbages	86	17	1,485	22	14	327	59	9	498	2,260	20	44,909
Lettuce	15	20	299	13	21	271	15	13	193	587	19	11,144
Tomato	609	20	12,452	82	28	2,336	149	44	6,628	2,323	25	57,330
Cauliflower	13	20	241	22	12	253	5	16	75	632	19	12,117
Squash	227	8	1,788	39	12	473	5	137	7	858	18	26,469
Cucumbers	4	16	72	1,729	18	3,167	94	13	717	857	14	11,557
Aubergines	46	19	940	3	24	60	1	19	9	1,506	17	26,000
Spinach	4	17	58	2	11	20	1	17	13	702	14	9,869
Pepper	268	8	1,989	146	13	1,814	29	14	397	970	15	14,056
Onions	38	13	466	45	25	1,131	22	5	113	106	20	2,126
Dry onion	281	14	4,012	19	14	260	160	21	3,416	1,971	17	32,575
Garlic	32	12	376	6	8	47	45	8	346	902	12	10,722
Carrots	74	13	952	18	25	432	46	21	935	507	19	9,749
maize	375	4	1,413	19	10	186	86	8	704	133	6	790

[a] From Sikora and Fernandez (2005) FAOSTAT database at: appjs.fao.org/faostat

The increase in the importance of vegetables is especially evident in countries with rapidly expanding populations, e.g. Africa and Asia, where large amounts of land near urban centres are devoted to vegetable production and where production since 1990 has expanded by 32 and 50%, respectively (Sikora and Fernandez, 2005).

The global production of tomato (fresh and processed) has increased by about 300% in the last four decades and is estimated of 100.5 million tons grown on 4,161,295 hectare. China, USA and Turkey are considered the three leading countries in producing tomato. Table 1.2 shows the top ten tomato-producing countries (Costa and Heuvelink, 2005).

Table 1.2 The top ten tomato-producing countries (Costa and Heuvelink, 2005 after FAO, 2003)

Country	Production (million ton)	Area harvested (ha)
China	25.9	1,105,153
USA	12.3	177,000
Turkey	9	225,000
India	7.4	520,000
Italy	6.9	130,932
Egypt	6.4	181,000
Spain	3.8	64,100
Brazil	3.6	59,766
Iran	3	112,000
Russian-Federal	2.2	160,000

Tomato is one of the most important crops in the Middle East where it is also a major income generating crop for small scale farmers. Jordan and Israel are the major producers of fresh or processing of tomato in this region (Costa and Heuvelink, 2005). In Palestine, tomato is grown in both large and small scale farms as an important vegetable and cash crop. Among all vegetables, tomato shares the highest total cultivated area (Table 1.3).

Table 1.3 The area and production of major vegetables in Palestine (1995-2000) Area/ Donum (1000m^2), Production/ Ton (1000kg)

Year	1995-1996		1996-1997		1997-1998		1998-1999		1999-2000	
Crop	Area	Prod.	Area	Prod.	Area	Prod.	Area	Prod.	Area	Prod.
Squash	27394	41546	29430	44284	31612	39251	26244	39119	28647	44827
Tomato	29831	122457	29203	140729	25918	153934	22896	172010	25392	196096
Cucumber	17555	91344	19187	106617	17516	99949	22000	104757	23099	130806
Watermelon	2895	7573	6605	15451	8381	19760	3220	13274	2688	7578
Cauliflower	7509	15972	8317	15150	8364	20394	8104	22240	7891	19208
Eggplant	6088	30715	9463	42973	7634	36339	8030	37088	8371	39716
Peas	3321	1242	2909	1556	5157	2414	2326	1037	--	--
Hot Pepper	4644	11410	5995	15739	5038	14650	3564	9945	4110	10967
Spinach	2135	3866	1784	2128	1697	2225	1332	2057	1360	2509
Onion	--	--	2765	6739	1475	3149	1709	3225	2101	4654
Carrot	648	1601	931	2992	118	3702	938	2615	723	1812
Radish	1461	3292	1558	3521	1074	2540	1341	2439	957	2070
Lettuce	694	1441	802	2365	972	2852	1151	3040	1018	3154
Paprika	353	1249	956	3805	773	3275	1334	4879	528	1131
Maize	--	--	--	--	655	838	548	1724	10295	11942

2. Importance of plant parasitic nematodes

At present, 24 genera of plant parasitic nematodes contain species that are economically important pests of crop plants. Other species and genera of plant parasitic nematodes may gain importance in the future as their host-parasite relationships become better understood. It has been estimated that some 10% of world crop production is lost as a result of plant nematode damage, which is one third of the losses generally attributed to pest and diseases (Whitehead, 1998).

Vegetables in both tropical and subtropical areas are highly dependent on good nematode control (Sikora and Fernandez, 2005). Plant damage increases with the number of nematodes feeding on the root system. Also yield and quality are reduced with increasing numbers of nematodes (figure 1.1), (Noling, 2005). Survival of plant parasitic nematodes depends on the availability of a suitable host plant (Sasser, 1979a, b), though they can survive unfavourable environments in several ways in the absence of the host. The eggs may resist low temperature or drought that juveniles or adults often cannot. Eggs may be retained in the dead body of the

females, which becomes a tanned, protective envelope or cyst. Some nematodes can withstand adverse environmental conditions by entering resting stages. The types of quiescence they enter include: low temperature (cryobiosis), high temperature (thermobiosis), lack of oxygen (anoxybiosis), osmotic stress (osmobiosis), and drying or dehydration (anhydrobiosis) (Wright and Perry, 2006)

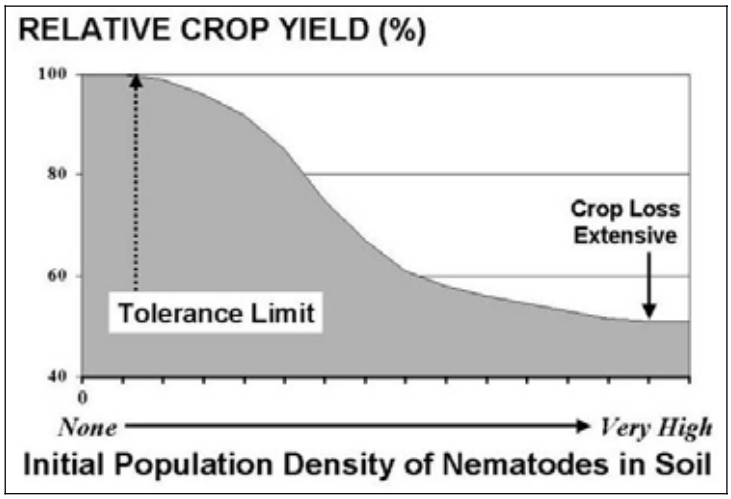


Figure 1.1 Typical nematode induced crop damage relationship in which crop yields, expressed as a percentage of yields that would be obtained in the absence of nematodes, decline with increased population density of nematodes in soil. The tolerance level is identified as the initial or minimal soil population density at which crop damage is first observed (after Noling, 2005).

The high reproductive capacity of plant parasitic nematodes is one of the features which make them such a significant pest, and also makes them very difficult to control. The life cycle of the majority of the important species takes only a few weeks at optimum temperatures, and each female has the capacity to lay hundreds of eggs (Stirling, 1991; Manzanilla-Lopez, 2004).

3. Root-knot nematode

Root-knot nematodes (*Meloidogyne* spp.) are an extremely important group of plant parasitic nematodes that have world-wide distributions, extensive host ranges and are able to interact with plant diseases to form complex disease syndromes (Hussey and Janssen, 2002; Manzanilla-Lopez, 2004; Karssen and Moens, 2006). Root-knot nematodes cause high losses in vegetables production worldwide. The estimated overall average annual yield loss of the world's major crops due to damage by plant parasitic nematodes is 12.3% (Sasser and Freckman, 1987). Species of *Meloidogyne* also cause severe damage to many other crop plants, i.e. legume, rice, etc. (Mai, 1985; Sikora et al., 2005). Tomato in particular, is heavily infected with *Meloidogyne* spp. in the tropics and subtropics as well as under greenhouse conditions in subtropical and temperate regions (Taylor and Sasser, 1978; Potter and Olthof, 1993; Whitehead, 1998; Sikora and Fernandez, 2005).

Four species, *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, count for 95% of all root-knot nematode infestations in agricultural land. Of these, *M. incognita* is the most economically important species. These highly successful pathogens cause an estimated average crop loss of 5% worldwide and are one of the major obstacles to production of adequate supplies of food in many developing countries (Hussey and Janssen, 2002).

Meloidogyne incognita is the most prominent and most widely distributed representative of this genus. It has a very wide host range, and can reproduce on more than 2000 plant species. *M. incognita* has 4 races identified by their host spectrum with different host preferences (Table 1.3) (Taylor and Sasser, 1978; Sasser, 1979a; Lamberti, 1979; Trudgill, 1997; Manzanilla-Lopez, 2004; Sikora and Fernandez, 2005).

Table 1.3 *Meloidogyne incognita* differential host test identification table

M. incognita races	Differential host plant cultivars*					
	Tobacco NC 95	Cotton Delta-pine 16	Pepper California Wonder	Watermelon Charleston Grey	Peanut Florrunner	Tomato Rutgers
Race 1	-	-	+	+	-	+
Race 2	+	-	+	+	-	+
Race 3	-	+	+	+	-	+
Race 4	+	+	+	+	-	+

*Tobacco (*Nicotiana tabacum*) cv NC 95; cotton (*Gossypium hirstutum*) cv Deltapine 16; pepper (*Capsicum frutescens*) cv California Wonder; watermelon (*Citrullus vulgaris*) cv Charleston Grey; peanut (*Arachis hypogaea*) cv Florrunner; tomato (*Lycopersicon esculuntum*) cv Rutger (Taylor and Sasser, 1978).

The life cycle of *Meloidogyne* spp. involves five developmental stages. Embryonic development in the egg results in formation of the vermiform first-stage juvenile (J1), which later molts into the J2 stage. The J2 hatch from the eggs by breaking the shell with repeated thrusting of their stylists, which then become the only infective stage. All other stages occur inside the root tissue (Mai and Abawi, 1987). Hatching of *Meloidogyne* eggs is temperature driven and occurs without requiring stimulus from plant roots, however, root stimulates sometimes stimulates hatching (Karssen and Moens, 2006).

Infective second stage juveniles (J2) migrate in the soil and are attracted to root tips where they penetrate behind the root cap. The juveniles migrate intercellulary in the cortical tissue to the region of the root where the vascular cylinder is differentiating. They inject secretory proteins produced in their oesophageal gland cells through the stylet into five to seven undifferentiated procambial cells to transform these root cells into very specialized feeding cells called giant-cells, which become the permanent feeding sites for the parasites throughout their life cycle (Dropkin, 1989; Trudgill, 1997; Jung and Wyss, 1999; Hussey and Janssen, 2002; Karssen and Moens, 2006).

Concurrent with the formation of giant cells, root tissue in the vicinity of nematode feeding undergo hyperplasia or hypertrophy. Together with the swelling female these changes at the cell level become apparent outside the root as galls or root-knots. Continuing developing in the root, the J2 goes through 3 moults in quick succession to the adult stage. The vermiform

male leaves the root, whereas the female develops into a globose adult (Dropkin, 1989; Jung and Wyss, 1999; Manzanilla-Lopez, 2004). Usually *M. incognita* females reproduces via mitotic parthenogenesis, whose eggs are deposited in a gelatinous matrix on the outer surface of the galled roots (Hussey and Janssen, 2002). Between 100-500 eggs are produced in the same gelatinous matrix. The first juvenile stage moult inside the egg to be released as a J2 whenever favourable conditions involving temperature and moisture exist (Dropkin, 1989).

The presence of galls on the root system is the primary symptom associated with *Meloidogyne* infection. The size and form of the gall depend on the species involved, the number of nematodes in the tissues, host and plant age. These galls disturb root function and affect nutrient and water uptake and consequently shoot growth which is suppressed by wilting especially under hot dry conditions as well as nutritional deficiency symptoms like chlorosis (Jenkins and Taylor, 1967b; Sikora and Fernandez, 2005).

Where seedling infection has taken place, numerous plants may die in the seedbed, seedlings do not survive transplanting, or weakened plants are strongly reduced in growth in the field (Sikora and Fernandez, 2005).

Figure 1.2 Galling symptoms of root-knot nematode *Meloidogyne incognita* race 3 infection on tomato roots

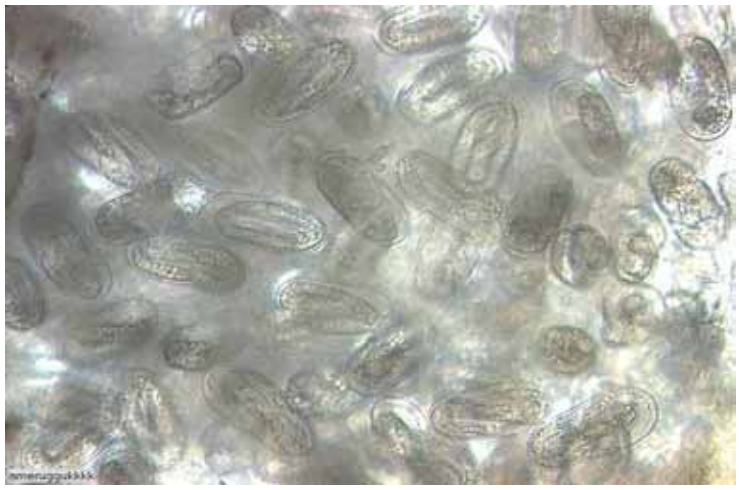


Figure 1.3 Eggs of root-knot nematode *Meloidogyne incognita* race 3 deposited in a gelatinous matrix or egg sac.

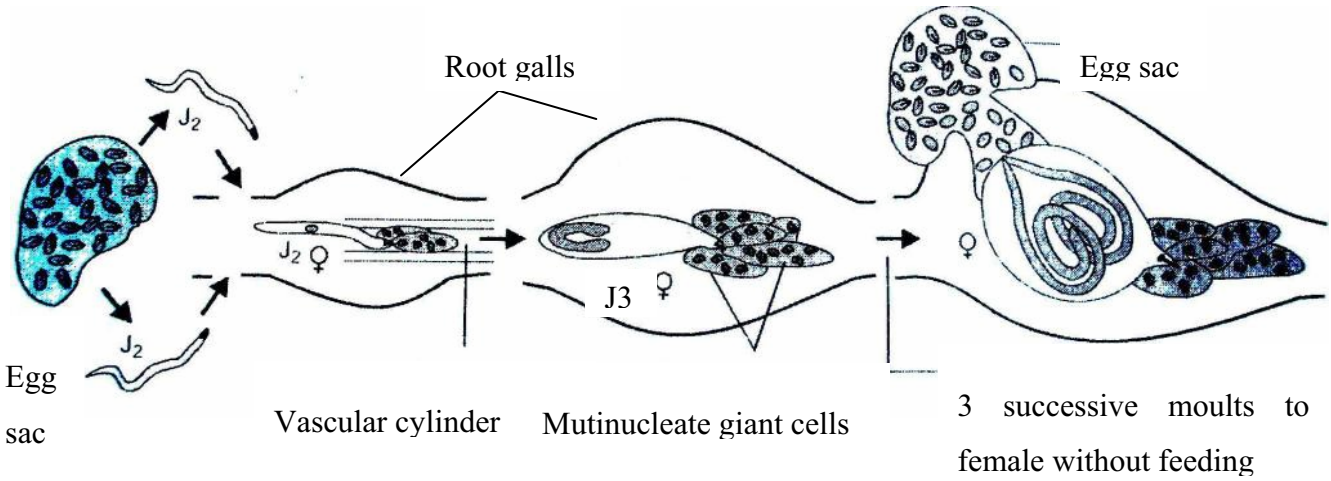


Figure 1.4 Life cycle of root-knot nematode (Jung and Wyss, 1999)

4. Root-knot nematode management techniques

The main aim of plant parasitic nematode management is to prevent significant losses of yield and quality in vulnerable crop plants, and in the long term, to keep nematode populations below the threshold level (Whitehead, 1998).

Species of *Meloidogyne* have been managed with varying degrees of success by a number of cultural, biological and chemical measures (Sikora and Fernandez, 2005) including crop rotation, use of resistant varieties, flooding, fallow, incorporation of organic amendments, soil solarization, steam heating, biological control agents and nematicides (Noling and Becker, 1994; Manzanilla-Lopez, 2004; Sikora et al., 2005).

Chemicals which paralyse or kill nematodes are collectively referred to as nematicides. The main mechanism of action of these chemical nematicides is associated with suppression of nematode mobility during the period when adequate concentrations are in the soil solution. However, use is often restricted due to high costs, microbial degradation and negative impacts of these nematicides on the environment. Over the past 20 years no new nematicides have entered the market place to replace the older highly toxic compounds still available.

Breeding for nematode resistance in vegetables is a superior alternative to nematicides and has been used extensively. Resistant plants prevent nematodes from completing their life cycles in the roots (Jenkins and Taylor, 1967; Whitehead, 1998). However, nematode resistance does not exist for many vegetables or is not incorporated into cultivars used in commercial production. Cultivar with resistance to *Meloidogyne* spp. also may breakdown when: 1) soil temperatures are high, 2) the roots are attacked by certain fungi and 3) when a cultivar is exposed to a species or a race to which it is susceptible.

Antagonist is a term for parasites, predators, pathogens, and other organisms that repel, inhibit or kill plant parasitic nematodes. Antagonists most likely to be effective for the biological control of nematodes are: plant-health promoting rhizobacteria, obligate bacterial parasites, fungal egg pathogens/parasites, predacious or trapping fungi, endoparasitic fungi, mutualistic fungal endophytes and endomycorrhizal fungi (Stirling, 1991; Sikora, 1992).

Therefore, finding and developing biological control agents as an additional nematode control component is important for integrated management (Kerry, 1987; Sikora, 1992; 1997; Viaene et al., 2006).

5. Biological control of plant parasitic nematodes

Biological control of plant parasitic nematodes has been defined as a reduction in nematode population density which is accomplished through the action of living organisms other than nematode resistant host plants. It occurs naturally, through the manipulation of the environment or following the introduction of antagonists (Jenkins and Taylor, 1967a; Stirling, 1991; Kloepper et al., 1992; Sikora, 1992).

Bacteria and fungi are the most abundant organisms in the soil and some of them have great potential as biological control agents against plant parasitic nematodes (Stirling, 1991; Sikora, 1992). Biological control of nematodes using antagonistic microorganisms is mediated through mechanisms such as predation, competition, antibiosis and parasitism (Stirling, 1991).

The need for biological control is important, because: 1) chemical nematicides which have been used effectively to control nematodes have been or are being withdrawn from the market because of health or environmental side effects, 2) crop rotation that has historically formed the basis of most management programs has become less attractive to growers because of economic pressure on land and demands for more specialization and intensification, 3) resistant crop varieties that have proven to reduce nematode infestation and have long lasting resistance, eventually breakdown due to virulent nematode pathotypes and 4) physical methods of control are costly or impractical under modern present day conditions (Kerry, 1987; Starr and Roberts, 2004).

Many different microorganisms have been used for the biological management of nematodes in soils. Bacterial and fungal agents have been used effectively to reduce a range of plant parasitic nematodes (Hoffmann-Hergarten et al., 1998; Kerry, 2000; Meyer et al., 2002; Hallmann et al., 2001; Chen and Dickson, 2004a, b).

In recent years, numerous experiments have shown that fungi (Kerry, 1987; Whitehead, 1998; Meyer, et al., 2000; Kiewnik and Sikora, 2003, 2004) and bacteria (Stirling, 1991; Hallmann, 2001; Siddiqui and Shaukat, 2004) applied to infested soil can lessen root-knot nematode infestation on susceptible plants. Some of these biocontrol agents have been used effectively at the field level and have been developed into commercial products such as Royal 350

(containing *Arthrobotrys irregularis*) and Bicon (containing *Paecilomyces lilacinus*) (Whitehead, 1998; Kiewnik and Sikora, 2003).

In addition, may other fungi notably *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*), a mutualistic endophytic fungus *Fusarium oxysporum*, and an obligate bacteria notably *Pasteuria penetrans*, have been reported to reduce nematode damage especially that caused by root-knot nematodes (Kerry et al., 1986; Hallman and Sikora, 1994a; Whitehead, 1998; Chen and Dickson, 2004a, b; Darban et al., 2004; Costa et al., 2006; Siddiqui et al., 2006; Viaene et al., 2006).

Although all of the above biocontrol agents are important for biocontrol, endophytic fungi probably have the greatest potential for effective nematode control. These fungi are found in the endorhiza of all major crops. By targeting the endorhiza as the site for application of biological control agents, efficacy is promoted by increasing the level of intimate contact between the biocontrol agent and the pest. In addition, by treating individual plants and not the soil the use of mutualistic endophyte reduces overall costs of the product to the farmer (Sikora et al., 2003).

Accordingly, high interest is being given to the study of non-pathogenic strains of *Fusarium oxysporum* in the biocontrol of soilborne pathogens (Alabouvette et al., 2001) as well as root-knot nematodes (Hallmann and Sikora, 1994b; Vu et al., 2004).

6. Non-pathogenic endophytic *Fusarium oxysporum* as biological control agent

Research has recently shifted from inundative treatment of soil with antagonists to the use of bacterial and fungal antagonists that reside endophytically in the endorhiza of the host plant (Pocasangre et al., 2000; Sikora et al., 2003). These endophytic microorganisms have the advantage of targeted application to seed and transplants, thereby reducing the amount of inoculum and overall costs of nematode management on a field basis and establishing the biocontrol agent in the potential infection zone of the host plant. (Harman, 1991; Sikora et al., 2000; Elzein et al., 2006).

Organisms that colonize healthy plant tissue without causing disease are known as mutualistic endophytes (Sikora, 1992; Hallmann and Sikora, 1994b; Viaene, et al., 2006). There are two types of endophytes organisms: obligate endophytes which cannot live outside a host plant, and facultative endophytes which can live on organic matter outside the plant.

Some strains of *Fusarium oxysporum* are known pathogens on certain crops and induce either root-rot or tracheomycosis (Alabouvette et al., 1998; Larkin and Fravel, 1999). However, the majority of strains are non-pathogenic saprophytes in soil (Alabouvette et al., 1998). Non-pathogenic strains of *F. oxysporum* have been used effectively for the control of plant pathogens in what is termed Cross-Protection (Kroon et al., 1991; Lemanceau and Alabouvette, 1991; Alabouvette et al., 1993) and is being used in the field to control *Fusarium* wilt of tomato.

Only a few species of *Fusarium* have been tested in the laboratory and the greenhouse for their potential as biocontrol agents against nematodes. *Fusarium* is a large genus including many species with a wide range of trophic adaptation. A number of *Fusarium* species have been isolated from females, cysts, egg masses, and eggs of nematodes.

Fusarium oxysporum is one of the most common species among soil fungi and grows endophytically in the endorhiza (Hallmann and Sikora, 1994a, b; Schuster et al., 1995; Sikora et al., 2003). The most predominant non-pathogenic fungus in the root system of most plants and in cultivated soil all over the world has been demonstrated to be *Fusarium*, with *F. oxysporum* the most common species (Alabouvette et al., 1998).

Interaction among strains of *F. oxysporum* can take place in a direct way in soil and in the rhizosphere. Direct interactions on the root surface, in the plant itself and indirect interactions through the plant resulting in a decrease of disease incidence have been reported (Alabouvette et al., 1998; Alabouvette et al., 2001).

It has been well established that pre-inoculation of a plant with an incompatible strain of *F. oxysporum* either a non-pathogenic strain or a pathogenic strain belonging to another forma specialis results in the reduction of the symptoms of wilt when the plant was, later on, inoculated with a compatible wilt inducing strain. This phenomenon was described as cross

protection or permutation. Today this phenomenon is considered as an expression of induced systemic resistance, a general mechanism of plant response to microbial infection or stresses from various origins (Alabouvette et al., 2001). Induced systemic resistance by non-pathogenic *F. oxysporum* strains has been considered as a mechanism responsible for mitigation of the wilt incidence caused by pathogenic *F. oxysporum* strains (Kroon et al., 1991; Lemanceau and Alabouvette, 1993; Fuchs et al., 1997; Larkin and Fravel, 1999).

Many non-pathogenic endophytic *Fusarium oxysporum* strains have been isolated from asymptomatic roots in nematodes infested fields where they grow without causing any visible damage to the plants. Such endophytic strains were isolated from many crops such as rice, maize, tomato or banana (Hallmann and Sikora, 1994a; Schuster et al., 1995; and Vu, 2005; zum Felde et al., 2005; Huong, 2006).

Strains of *F. oxysporum* also have been shown to reduce root-knot nematode infection of tomato (Hallmann and Sikora, 1994b; Sikora et al., 2003). Gall formation was reduced between 50 and 75% when the strain *F. oxysporum* 162 was applied to the substrate used for seedling production and these seedlings challenged later with root-knot nematode. This strain also increased root growth significantly.

F. oxysporum has also been shown to reduce lesion nematodes *Pratylenchus zeae* on maize (Kimenju et al., 1998) and the burrowing nematodes *Radopholus similis* on banana (Niere et al., 1999; Pocasangre, 2000; zum Felde et al., 2005). Banana tissue culture transplants reared in substrate inoculated with non-pathogenic *F. oxysporum* and challenged 5 weeks later with *R. similis* had 50% lower nematode densities in their roots than the untreated control (Sikora et al., 2000; Vu, 2005). Sikora and Pocasangre (2004) demonstrated a reduction of 86% in *R. similis* densities in banana inoculated with endophytic fungi. Reductions in *R. similis* damage in both greenhouse trials and in field trials (Schuster et al., 1995; Niere et al., 1999; Pocasangre 2000; zum Felde et al., 2005) have been reported.

7. *Trichoderma* as biological control agents

Fungal species of the genus *Trichoderma* are worldwide in occurrence and easily isolated from soil, decaying wood, and other forms of plant organic matter (Howell, 2003). *Trichoderma* species are very common fungal biological control agents in soil and have been extensively researched and employed in biocontrol studies throughout the world. *Trichoderma* species have been successfully used for pathogen control on a number of vegetables crops (Windham et al., 1986; Harman et al., 1989; Meyer et al., 2000) as well as on cereal (Pant and Pandey, 2002; Pandey et al., 2003). *Trichoderma* isolates are well known for their antagonistic activity over other fungi (Papavizas, 1985; Elad, 1994; Bandyopadhyay and Cardwell, 2003) and have proved to be a very potent biocontrol agent against several soilborne plant pathogenic fungi under both greenhouses and field conditions (Sivan and Chet, 1993).

Trichoderma-induced increased growth response has been reported for various plant species, including maize (Windham, 1989), tomato, cucumber, melon, tobacco (Windham et al., 1986; Kleifeld and Chet, 1992), pepper (Inbar et al., 1994), chickpea (Pandey et al., 2003).

Until recently, *Trichoderma* was believed to achieve biocontrol mainly by direct effects on fungal pathogens, particularly via mycoparasitism, antibiosis and competition (Tronsmo, 1996; Howell, 2003; Pandey et al., 2003).While these mechanisms are important, indirect effects over the plant are now thought to be also important.

Trichoderma isolates have been evaluated recently for their efficiency to control the root-knot nematode on vegetables and especially on tomato (Windham et al., 1989; Spiegel and Chet, 1998; Meyer et al., 2000; Sharon et al., 2001). Tomato root explant cultures of *M. incognita* treated with *Trichoderma virens* culture filtrate had 42% fewer eggs and J2 per g of roots than explants treated with control medium that had not been inoculated with *T. virens* (Meyer et al., 2000).

In another study, Spiegel and Chet (1998) studied 2 *Trichoderma* isolates against the root-knot nematode *M. javanica.* They showed that *Trichoderma* isolates were able to reduce

significantly both the root-galling index and number of eggs per g root in a short term greenhouse experiment but not in long-term experiments.

Pandy et al. (2003) concluded that the number of galls of *M. incognita* on chickpea roots was decreased whenever the roots were inoculated with *T. viride* and this reduction was related to the fungal concentration used. Windham et al. (1989) studied the reproduction of *M. arenaria* on root-knot susceptible and resistant maize cultivars and showed that the reproduction of the nematode on the root-knot susceptible maize was significantly reduced.

8. Scope of the study

The main goal of this research was to elucidate the mechanisms of action involved when the non-pathogenic fungus *Fusarium oxysporum* strain 162 and different strains of *Trichoderma* were used for biocontrol of the root-knot nematode *Meloidogyne incognita* on tomato. The objectives of these investigations were to determine:

1- Biocontrol activity of *Trichoderma* strains against *Meloidogyne incognita in vitro* and *in vivo*.

2- The efficacy of the non-pathogenic fungus FO162 as a potential biological control agent against *M. incognita*.

3- The ability of the endophytic fungus FO162 to enhance plant growth promotion.

4- The mechanisms of action of the *F. oxysporum* 162 against *M. incognita.*

5- Colonisation efficiency of *F. oxysporum* 162 in *Fusarium* susceptible and wilt resistant varieties.

6- The biocontrol activity of *F. oxysporum* 162 against *M. incognita* on a wide spectrum of tomato varieties resistant and susceptible to *F. oxysporum*.

Chapter two
GENERAL MATERIALS AND METHODS

Materials and methods generally used in this study are described below. Additional techniques applied in individual experiments are described within the respective chapter.

1. Fungal isolates

1.1 Origin

Four *Trichoderma* isolates from different countries, were tested against *M. incognita* (table 2.1). The non-pathogenic endophytic fungus *F. oxysporum* strain 162 was originally isolated from the cortical tissue of surface sterilised tomato roots (Hallmann and Sikora, 1994a).

Table 2.1 The biocontrol agents used in this study.

Symbol	Isolates	Origin
Tv	*Trichoderma viride*	Palestine
Th1	*Trichoderma harzianum*	Palestine
Th2	*Trichoderma harzianum*	Israel
Th3	*Trichoderma harzianum*	Thailand
FO162	*Fusarium oxysporum 162*	Kenya

1.2 Mass production of the different biocontrol agents

Fungal isolates were cultured and reared on Potato Dextrose Agar (PDA) containing 150 mgl^{1} streptomycin and 150 mg l^{-1} of chloramphenicol (DIFCO Company) and placed in an incubator at 24 ºC between 7 to14 days. Spores and microconidia from these initial plates were stored in micro-bank tubes (CRYOBANK^{TM} , MASTE Group Ltd., Merseyside, UK) at –80 ºC to avoid mutation. Fungal propagation for all further experiments was initiated with spores from this stock. Inoculum was produced by culturing the fungi on PDA plates. After 1 to 2 weeks, depending on level of spore production, the mycelia and spores were scratched from the surface suspended in water and sieved through 3 layers of cheese cloth or through a

50 µm aperture sieve and used immediately in the experiments after adjusting the concentrations. In some tests 10 mm diameter discs taken from 7 days old PDA cultures were added to flasks containing 200 ml Potato Dextrose Broth (DIFCO Company) and incubated 7-10 days under continuous agitation in an incubator at 25ºC. After fermentation, fungal mycelia were separated from the broth by passing them through cheese cloth. All spore solutions were centrifuged at 5000 rpm at 20ºC for 20 min, the nutrient media discarded and the spores were then re-suspended in tap water and directly used.

2. Nematodes

2.1 Nematode inoculum

Meloidogyne incognita race 3 originating from Florida was used in all the experiments. Nematode inoculum was multiplied on the tomato plant cv. Furore grown in a greenhouse at 27±5 °C. The plants were grown in a large container (150x80x40 cm) filled with a sterilized sand:soil substrate (2:1, v/v), respectively. Tomato seedlings, 3-4 weeks old, were transplanted into the substrate and fertilized weekly with a 0.3% fertilizer solution (N:P:K 14:10:14). Removed tomato plants for nematode extraction were replaced by 3-4 week old plants to maintain nematode inoculum production levels.

Eggs were extracted from 2 month old galled tomato roots using 1.5% NaOCl as described by Hussey and Barker (1973). The plants were uprooted and washed with water to remove adhering soil particles. The roots were then cut into 1-2 cm pieces and macerated 2 x 10 seconds in a small amount of water at high speed using a warring blender (Bender and Hohbein). The macerate was filled into a flask containing 600 ml of a 1.5% NaOCl solution and manually shaken for 3 minutes to free eggs from the gelatinous matrix. The egg suspension was poured over a sieve combination of 250 µm; 100 µm, 45 µm and 25 µm aperture and gently rinsed with tap water to remove excess chlorine. Eggs collected on the 25 µm aperture sieve were either used directly in experiments requiring egg inoculum or transferred into a beaker and agitated for 7 to 10 days in the dark with simultaneously supplied oxygen from an aquarium pump to induce juvenile hatching. Freshly hatched J2 were separated from the un-hatched eggs using a modified Baermann technique. The juveniles in the resulting water suspension were adjusted to 1000 J2/ml and used as inoculum for the

experiments. For the experiments which were inoculated with eggs, the eggs were counted and the number adjusted to 5000 eggs/ml and used as inoculum.

2.2 Nematode sterilization

Sterilised J2 were used in the *in vitro* experiments for screening activity of the different biocontrol agents against *M. incognita*. Nematodes were collected on a 25 µm sieve, then transferred to a 15 ml tube and finally centrifuged for 3 min at 3000 rpm, which caused the nematodes to form a pellet at the bottom of the tube. Under the laminar-flow hood, the supernatant was removed with a sterile pipette. With another sterile Pasteur pipette, 2 ml of a 2000 ppm streptomycin sulphate solution was added to the tube and left for one hour. The suspension was then centrifuged for 5 min at 3000 rpm. After removing the supernatant, the nematodes in the tube were washed three times with sterilized water to remove the excess streptomycin sulphate. Each time the tube was filled to the 10 ml level with sterilised water and then centrifuged for 5 min at 3000 rpm.

3. Culture media and reagents

Potato Dextrose Agar PDA (DIFCO)

24	g	Potato Dextrose Broth
18	g	Agar (DIFCO)
1000	ml	Distilled water

150 ppm streptomycin sulphate and 150 ppm chloramphenicol were added after cooling

Dilute Potato Dextrose Agar 10% PDA (DIFCO)

2.4	g	Potato Dextrose Broth
30	g	Agar (DIFCO)
1000	ml	Distilled water

150 ppm streptomycin sulphate and 150 ppm chloramphenicol were added after cooling

Potato Dextrose Broth PDB (DIFCO)

6	g	Potato Dextrose Broth
250	ml	Deionised distilled water

Phloxine B (MERCK)

15	mg	Phloxine B
1000	ml	Tap water

Fuchsine acid (MERCK)

2 g fuchsine acid powder + 198 ml water (1% fuchsine acid)

Lactic acid solution:

1750	ml	Lactic acid
126	ml	Glycerine
124	ml	Tap water

1 % of the fuchsine acid added to lactic acid solution (Sikora and Schuster, 2000)

4. Statistical analysis

Data were analysed according to standard analysis of variance procedures with the SPSS 14 program for windows. Differences among treatments were tested using one way analysis of variance (ANOVA) followed by Tukey Test for mean comparison if the F-value was significant. Statistical differences referred to in the text were significant at (P <0.05). However, T-test was used for comparing 2 treatments using SigmaStat 3.1.

The data in chapter 6 were analyzed using mixed procedure of SAS (2005). Means of levels of the significantly affecting factors were compared using Tukey's studentized range test with SAS.

Chapter Three

USE OF *TRICHODERMA HARZIANUM* AND *TRICHODERMA VIRIDE* FOR THE BIOLOGICAL CONTROL OF *MELOIDOGYNE INCOGNITA* ON TOMATO AND THEIR MODE OF ACTION

1. Introduction

Biological control of soilborne plant pathogens and plant parasitic nematodes by antagonistic microorganisms is a potential non-chemical means of plant disease control, (Papavizas, 1985; Kerry, 1987; Stirling, 1991; Elad et al., 1993). A wide range of bacteria (Hallmann et al., 2001) and fungal agents (Spiegel and Chet, 1998; Kerry, 2000; Meyer et al, 2001) have been used to reduce a range of plant parasitic nematodes.

Some species of *Trichoderma* have been used widely as biocontrol agents against soilborne plant diseases (Harman, 1991; Inbar et al., 1994; Tronsmo, 1996; Rabeendran et al., 1998; Whipps, 2001). *Trichoderma* isolates have been used successfully to control the damage caused by soilborne pathogens in greenhouses and under opened field conditions (Papavizas, 1985). *Trichoderma* also have been shown to have activity toward root-knot nematode (Windham et al., 1989; Sharon et al., 2001). Some *Trichoderma* isolates were reported to enhance plant growth and also reduced root-knot nematode damage (Windham et al., 1989; Meyer et al., 2001).

Fungal pathogens are killed by the releases of toxic compounds (e.g. the antibiotics gliotoxin, gliovirin, and peptabiols) and a battery of lytic enzymes, mainly chitinases, glucanases, and proteases by species of *Trichoderma*. These enzymes facilitate penetration into the host and the utilization of host nutrients (Lorito et al., 1996).

Direct pathogenicity of fungal biocontrol agents is one of the main mechanisms responsible for plant parasitic nematode control (Kerry, 1987; Stirling, 1991). However, secondary

metabolites from fungi also contain toxins which are toxic to plant parasitic nematode (Hallmann and Sikora, 1996; Sikora et al., 2003). Culture filtrate of different biocontrol agents effective against plant parasitic nematodes, was previously studied by many researchers (Reißinger, 1995; Hallmann and Sikora, 1996) and more recently by Meyer et al. (2004) and Vu, (2005).

Several attempts have been made to use different species of *Trichoderma* for the control of plant parasitic nematodes with unsatisfactory levels of control (Spiegel and Chet, 1998; Dababat et al., 2006).

A number of *Trichoderma* isolates are now used commercially for control of fungal pathogens in the soil. These isolates, however, have not been tested for root-knot nematode control. The aims of the following experiments were to:

1. Determine the effect of *Trichoderma* as a biocontrol agent for control of the root-knot nematode *M. incognita.*

2. Test the effect of time of inoculation on control efficacy.

3. Study the effect of the different isolates on plant growth promotion.

4. Investigate direct parasitism of *Trichoderma* towards *M. incognita*.

5. Examine the influence of secondary metabolites on *M. incognita* mobility and mortality.

2. Materials and Methods

2.1 Influence of duration of exposure of *Trichoderma* to *Meloidogyne incognita* on biocontrol

2.1.1 Soil treatment at planting

Tomato seeds of cultivar Hellfrucht Frühstamm were sown in commercial style 70 plug seedling trays, with each plug measuring 4 x 4.5 x 2.5 cm. Seedlings were then propagated for 3 weeks in the greenhouse, before being transplanted into new pots (7x7x8 cm) containing 400g of a mixture of sterilized sand and soil 2:1 respectively. One week after transplantation the seedlings were inoculated with one of the following fungi: *T. viride* (Tv), *T. harzianum* (Th1), *T. harzianum* (Th2) or the positive control *F. oxysporum* (FO162). The endophytic non-pathogenic *F. oxysporum* 162 demonstrated to be highly antagonistic to root-knot nematodes was used in this study as a positive control (Hallmann and Sikora, 1994a). Each seedling received 1 ml of a liquid suspension of the *Trichoderma* isolates containing 5 x 10^6 spores ml^{-1} or 1.5 x 10^7 spores ml^{-1} of FO162. The inoculum was injected 2cm deep into the rhizosphere using 3 holes made around the stem base with a plastic rod. The absolute controls were treated with tap water. The pots were then immediately inoculated with a 3 ml tap water suspension containing 650 J2 of *M. incognita.* The inoculum was also injected into 3 holes roughly 2 cm deep around the stem base. Nematode and fungi were produced for this experiment as outlined in (chapter 2).

The experiment consisted of six treatments: 1) Tv + Mi, 2) Th1 + Mi, 3) Th2 + Mi, 4) FO162 +Mi, 5) Mi, and 6) absolute control. The treated pots were incubated in the greenhouse at 22ºC ±5 with 16 hours of supplemental artificial light per day. The plants were watered and fertilized with 2 g per liter water (N:P:K 14:10:14) to insure proper plant growth. The plants were periodically examined for phytotoxicity.

The experiment was terminated 8 weeks after fungal and nematode inoculation. Fresh shoot weight as well as plant height from the base of the stem to the growing point was measured. The roots were removed, washed free of soil and stained in 0.015% Phloxine B for 20 minutes to facilitate egg mass counting. The number of galls and egg masses per plant was then determined.

Trichoderma re-isolation from the soil was evaluated by taking soil samples from 5 replicates. Samples were taken from 3cm deep using cork borer. A total of 15 g soil of each treatment was collected. Trichoderma were isolated from the soil by dilution plating technique on 9 cm diameter Petri dishes, using PDA medium. The Petri dishes were incubated at 25° in the dark. One week after plating, pure culture was made to check for the presence of inoculated isolates. Furthermore, 12 root sections were taken from all plants inoculated with FO162 and from the absolute control, and used for re-isolation of FO162 to determine the level of endophytic colonization. Endophytic colonization was determined after sterilization of the root surface with 1.5% NaOCl for three minutes, followed by three rinses in sterilized water. Root sections of approx. 0.5 cm length and of a uniform size of approx. 0.5-1mm diameter were removed and placed onto PDA Petri plates containing 150 mg l^{-1} streptomycin and 150 mg l^{-1} chloramphenicol. Successful re-isolation was confirmed when the growth characteristics of the fungus and its colour on PDA, as well as the shape of the conidia corresponded with that of the original isolate.

2.1.2 Soil treatment before transplanting

The same general procedures mentioned above were followed in this test. However, the soil in the pots was treated with the biocontrol agents and 5000 nematode eggs one week before transplanting the tomato seedlings. In this experiment *T. harzianum* (Th3) was also tested in addition to the other fungal isolates used in the first experiment.

2.2 Pathogenicity of *Trichoderma* isolates toward *M. incognita* in soil in the absence of tomato

This test was designed to determine direct pathogenicity of the different *Trichoderma* isolates against *M. incognita* J2 in soil in the absence of tomato. A 1 ml spore suspension of *Trichoderma* at 5x 10^6 spores or FO162 containing 1.5x 10^7 spores was inoculated into 100 cm^3 pots filled with 100 g sterile sand obtained by sieving through 500 µm aperture sieve. Fungal and nematode inoculation was done as in experiment 3.1.1. Tap water was used as a control. Pots were placed in the greenhouse at 24°C, and watered daily. One day after fungal treatment, a 1 ml solution containing 1000 J2 of *M. incognita* were added to each pot in three 2 cm deep holes. The level of mortality was determined by extracting the J2 from the sand by

adding the sand to a 2 liter bottle filled with tap water. The bottle was agitated and the supernatant then passed through a 20 µm sieve. The nematodes collected on the 20 µm sieve were examined and active and inactive nematodes counted. The experiment was repeated a second time.

2.3 *In vitro* effects of metabolites of *Trichoderma* on mobility and mortality of *M. incognita*

Trichoderma isolates were first cultured on PDA in an incubator at 25°C for one week. Five 0.5 cm plugs of PDA with fungal mycelia were added to flasks containing 200 ml of PDB. The flasks were shaken in an incubator at 25°C for one week. Fungal mycelia were removed from the broth by filtration through 3 layers of cheese cloth. The suspension was then centrifuged at 5000rpm for 20 min at 20°C to remove small sections and spores. The supernatant was filtered through a combination of 0.45 µm and 0.22 µm pore size micro-filters. 0.3 g of streptomycin sulphate was added to the solution so that the final antibiotic concentration was 150ppm to prevent microbial growth. FO162 used as standard was produced in the same manner as in (chapter 2 section 1.2). Sterile water as well as filtrate from 5 un-inoculated PDA pieces in PDB medium was used as the absolute control. The experiment was conducted under sterile conditions and set up in 6 multi well-trays. For each treatment, 3 ml of sterile metabolite solution was combined with 3 ml sterile water containing 1000 sterile J2 in each well for a final concentration of 50%. A 90% solution was prepared by combining 5.4 ml of metabolite solution with 0.6 ml of sterile water containing 1000 sterile J2 of *M. incognita*. The multi well-trays were stored in an incubator at 25°C and mobility of the J2 was measured after 24 hours at a concentration of 90% and after 4 days at the 50% concentration. A 1 ml suspension of J2 from each well was transferred to a counting chamber and mobility of 100 nematodes was recorded. Nematodes which were inactivated were added to tap water for 24 hours and those which were still immobile and straight were considered as dead and mortality calculated.

3. Results

3.1 Influence of duration of exposure of *Trichoderma* to *Meloidogyne incognita* on biocontrol

3.1.1 Soil treatment at transplanting

Simultaneous treatment of the soil at transplanting with *Trichoderma* and *M. incognita* resulted in a reduction of up to 19.5 % in nematode galling. A significant reduction in gall formation was obtained with *Trichoderma harzianum* (Th1) when compared to the control. However, in both experiments, the FO162 standard gave higher levels of control when compared to all *Trichoderma* isolates (figure 3.1). The suppressive effect of the fungi on egg mass production, which is a measure of nematode development over time, was confirmed only for FO162. *Trichoderma* isolates did not reduce egg mass number (figure 3.1).

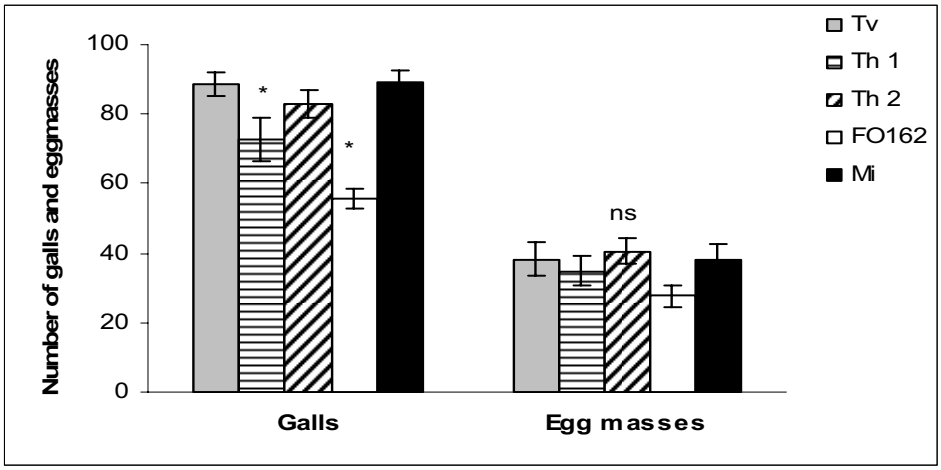
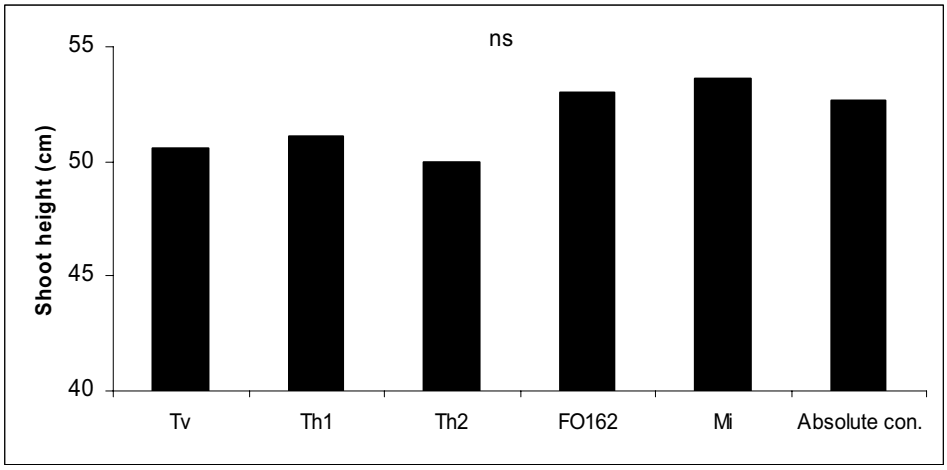


Figure 3.1 Influence of simultaneous soil treatment of *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 with *Meloidogyne incognita* on gall and egg mass number 8 weeks after nematode inoculation at the time of transplanting tomato. Means with (*) are significantly different from the control *Meloidogyne incognita* based on Tukey Test ($P \leq 0.05$; n = 10).

Treating the soil simultaneously at transplanting with *Trichoderma* or *F. oxysporum* 162 and *M. incognita* had no significant effect on shoot height (figure 3.2 a). Shoot weight, however, slightly increased when the plants were inoculated with *Trichoderma* isolates Th1 and Th2 or FO162 compared to the absolute control (figure 3.2 b).

A



B

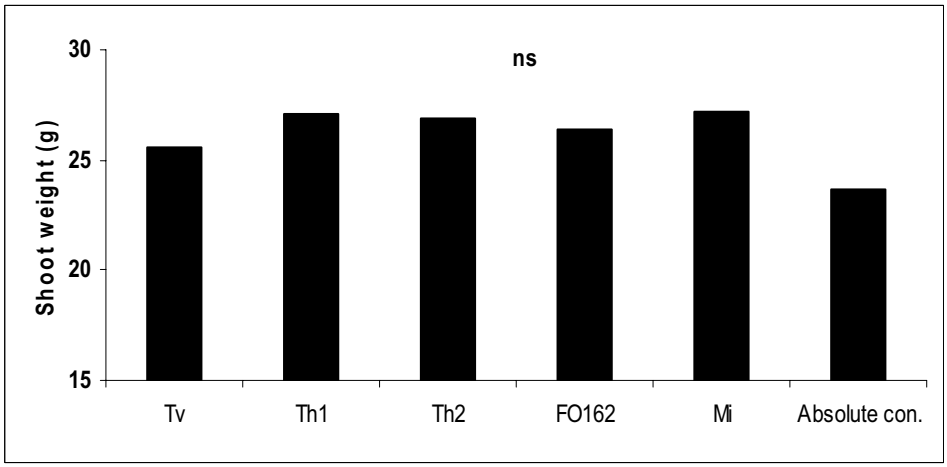


Figure 3.2 Influence of simultaneous treatment of soil with *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 with *Meloidogyne incognita* on shoot height (A) and shoot weight (B) 8 weeks after nematode inoculation. NS: no significant difference based on Tukey Test ($P \leq 0.05$; n = 10).

3.1.2 Soil treatment before transplanting

Inoculating the soil with *Trichoderma* and *M. incognita* one week before transplanting tomato seedlings resulted in reductions of up to 30.8 % in nematode galling. The differences however, were not significant due to high variation. FO162, on the other hand, caused a significant reduction of 44.5% in nematode galling (figure 3.3).

Trichoderma viride caused a significant reduction in egg mass number by 20 percent. Whereas, FO162 reduced egg mass formation 50 percent over the absolute nematode control (figure 3.3). In contrast to the treatment at planting where no effect on egg mass number was observed, most of the *Trichoderma* isolates were able to induce a slight reduction in egg mass number when added to the soil one week before transplanting (figure 3.3).

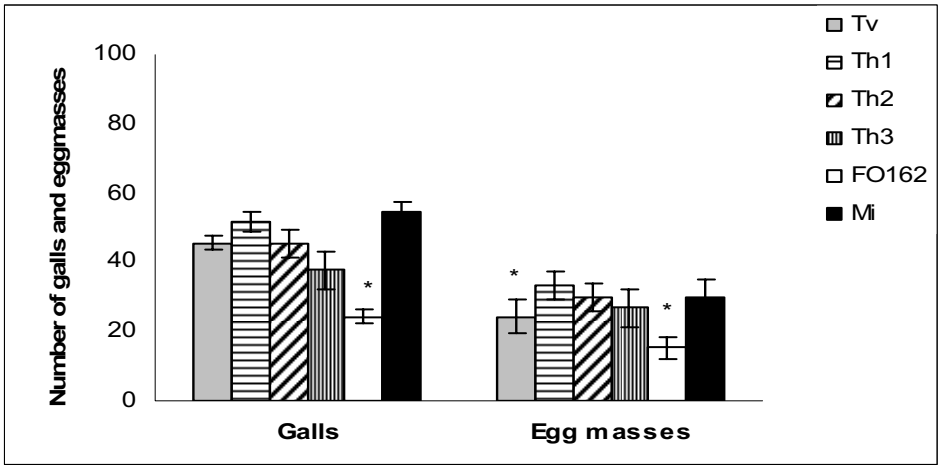
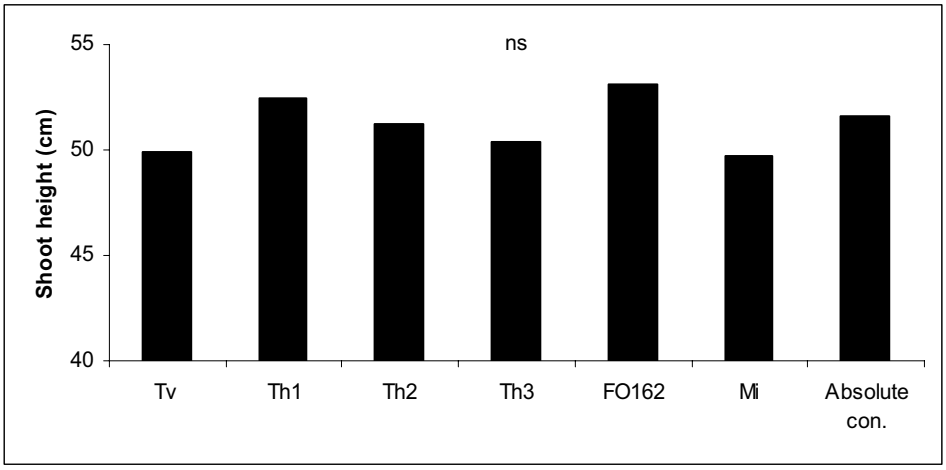


Figure 3.3 Influence of soil pre-treatment with *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 on gall and egg mass number of *Meloidogyne incognita* 9 weeks after nematode inoculation of tomato. Means with (*) are significantly different from the control *Meloidogyne incognita* based on Tukey Test ($P \leq 0.05$; n = 10).

The results of pre-inoculating soil with *Trichoderma* and the nematode before planting had no significant effect on shoot height (figure 3.4 a), Th1 and FO162 produced a slight increase in height. Shoot weight increased slightly but not significantly when the plants were inoculated with the *Trichoderma viride* and *Trichoderma harzianum* (Th2) as well as with FO162 when compared to both the absolute and nematode controls (figure 3.4 b).

A



B

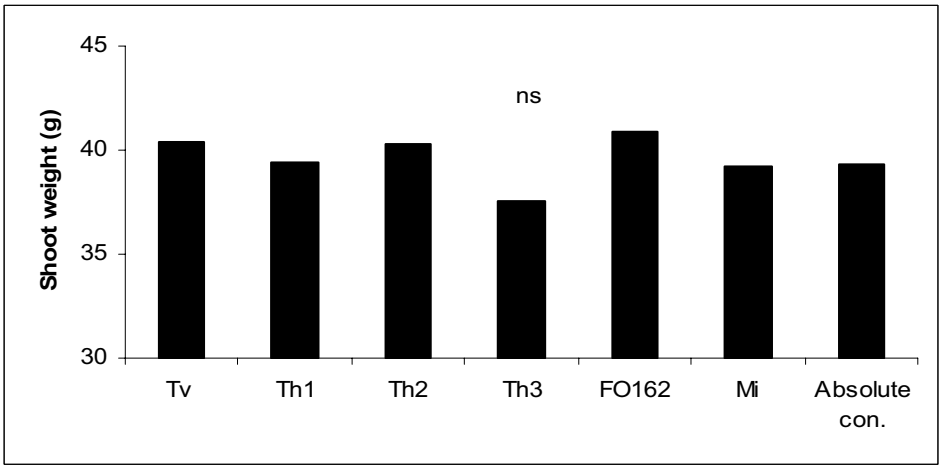


Table 3.4 Influence of soil pre-treated with *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 with *Meloidogyne incognita* on shoot height (A) and shoot weight (B) 9 weeks after fungal inoculation. NS: no significant difference based on Tukey Test ($P \leq 0.05$; n = 10).

3.2 Pathogenicity of *Trichoderma* toward *M. incognita* in soil in the absence of tomato

Nematodes mortality as affected by the three *Trichoderma* isolates ranged from 36-40 percent and was significantly higher when compared to the nematode controls (figure 3.5). There were no significant differences in the activity between the *Trichoderma* isolates. FO162 when compared with the *Trichoderma* isolates gave superior nematode control of up to 80%.

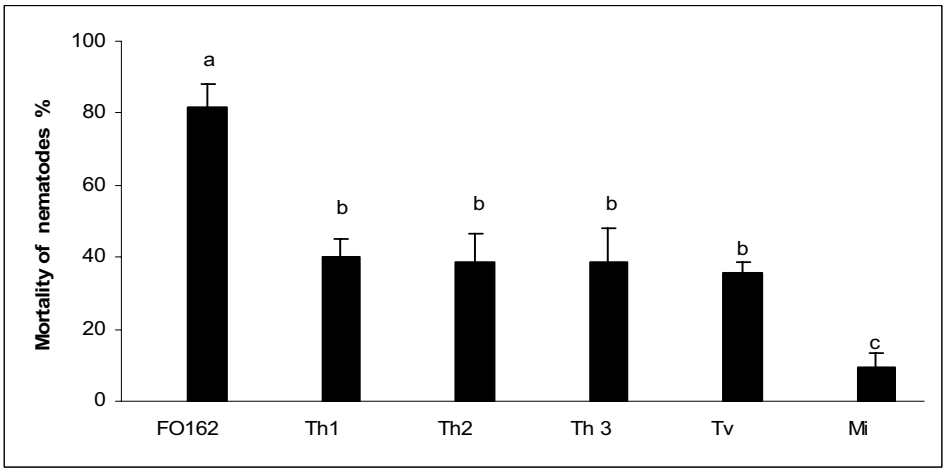


Figure 3.5 Effect of *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 on *Meloidogyne incognita* mortality in soil in the absence of tomato. Means with different letters in the same column are significantly different based on Tukey Test ($P \leq 0.05$; n = 8).

3.3 *In vitro* effects of metabolites of *Trichoderma* on mobility and mortality of *M. incognita*

Exposing the nematode to culture filtrates of *Trichoderma* at a concentration of 90% significantly decreased the mobility of *M. incognita* J2 after 24 hours when compared to the water control. Mortality after testing recovery of inactive nematodes in tap water was still high and ranged between 14.3- 38.3% in the treatments compared to 3% in the water control (figure 3.6).

Significant differences between the different biocontrol agents were found. The highest level of nematode mortality was obtained by *Trichoderma harzianum* 3, which produced nematode mortality of 38%.

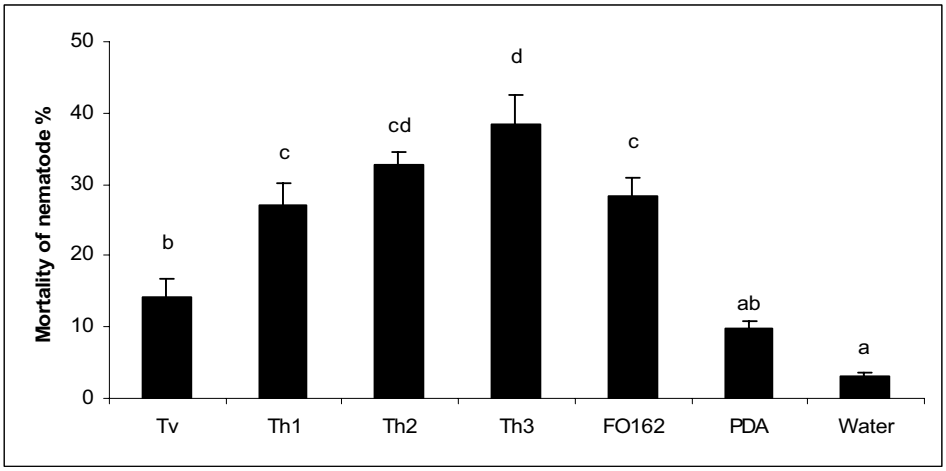


Figure 3.6 Effect of metabolites of *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 on *Meloidogyne incognita* mortality after 24 hours *in vitro* (90% concentration). Means with different letters in the same column are significantly different based on Tukey Test ($P \leq 0.05$; n=6)

Culture filtrates of all fungi at a concentration of 50% decreased nematode mobility significantly after 4 days over the water control. Nematode immobility ranged from 10 to 28% among the biocontrol agents compared with 7.8 % and 1.3% in the media and water controls respectively (figure 3.7). No significant differences among the *Trichoderma* isolates were detected. FO162 gave the highest level of inactivation after 4 days. All *Trichoderma harzianum* isolates had a significant effect on nematode mobility at concentration of 50% and after 4 days but not *Trichoderma viride*, however, the level of control was far below that of the FO162 standard.

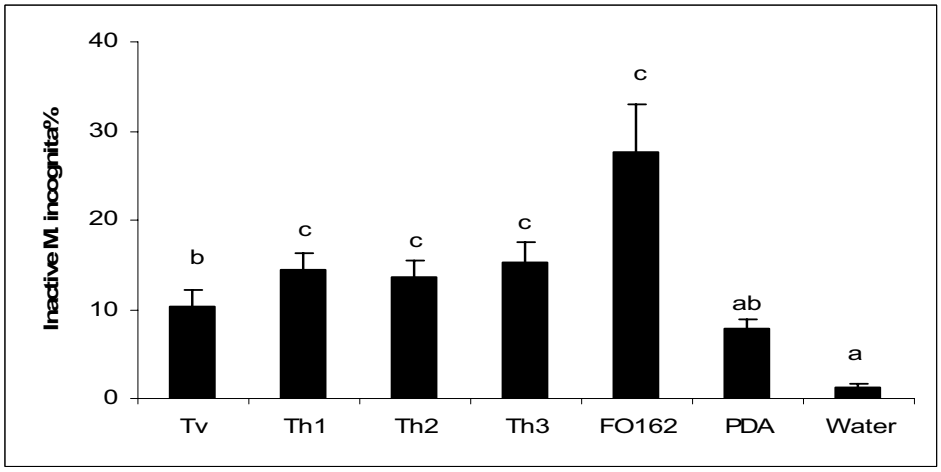


Figure 3.7 Effect of metabolites of *Trichoderma harzianum*, *Trichoderma viride,* or *Fusarium oxysporum* 162 on *M. incognita* mobility after 4 days *in vitro* (50% concentration). Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n=6)

4. Discussion

4.1 Influence of duration of exposure of *Trichoderma* to *M. incognita* on biocontrol

Treatment of the soil with the *Trichoderma* isolates at the time of transplanting resulted in a small reduction in nematode galling. However, galling was significantly reduced when treatment took place one week before transplanting - allowing for extended exposure of the nematode to the antagonist. In both simultaneous inoculations one week before or at transplanting, treatment of the plants with the standard biocontrol agent FO162 reduced the number of egg masses, while *Trichoderma* isolates gave only slightly reductions. FO162 had a greater impact on nematode galling when compared to the *Trichoderma* isolates.

Similar results were obtained by other researchers with *Trichoderma*. Sharon et al. (2001) reported that *Trichoderma harzianum* reduced galling of root-knot nematode *Meloidogyne javanica* on tomato plants. Spiegel and Chet (1998) used different *Trichoderma* isolates against the root-knot nematode *M. javanica*, and the results showed that a decrease in root-galling index as well as eggs per gram of root were achieved when nematode-infested soils were pre-exposed to the *Trichoderma* preparations in short term trials. Similar results were obtained by Pandey et al. (2003) using different treatments of *Trichoderma viride* against *M. incognita* in chickpea. All *Trichoderma viride* treatments decreased galling and the final nematode population densities of *M. incognita* in both field and pots experiments as the level of the *T. viride* increased. Number of eggs and second-stage juveniles (J2) per gram root were significantly lower with fungal treatment.

In the current study inoculating the seedlings with *Trichoderma* did not have a consistent positive effect on plant growth. Neither shoot weight nor plant height was clearly affected. The results are similar to those of Sankaranarayanan et al. (2002) who showed that maximum plant height was reached in the non-inoculated control plants followed by those treated with the biocontrol agents. Similar results also were obtained by Meyer et al. (2001) with *Trichoderma*. Conversely, Windham et al. (1986), Kleifeld and Chet (1992), Spiegel and Chet (1998), Sharon et al. (2001), and Pandey et al. (2003) reported a stimulatory effect of *Trichoderma* spp. on plant growth.

Root weight following *Trichoderma* inoculation was not significantly affected. Treatment with *Trichoderma* did not affect plant growth in studies by Meyer et al. (2001). However, Windham et al. (1986), Spiegel and Chet (1998) and Pandey et al. (2003) reported a positive effect of *Trichoderma* spp. on plant growth parameters when applied to the plants. The increase in root weight in the present studies was caused by the size and number of the galls formed on the roots.

All *Trichoderma* isolates could be re-isolated from rhizosphere soil. Re-isolation of the fungi from endorhiza was confirmed for FO162 and not for the *Trichoderma* isolates. The addition of the fungi did not cause disease symptoms on the plants.

The level of control with *Trichoderma*, obtained in the present tests, using short exposure times to juveniles, did not prove effective for further field development of these isolates as a commercial product. One week pre-exposure or simultaneous treatment at transplanting gave small to moderate levels of control, not sufficient for practical application.

The level of biocontrol could be improved significantly, if the nematode is exposed to the *Trichoderma* for longer periods of time before transplanting. For example, treatment during the preparation of raise-beds in vegetable production would give the antagonist a 5 week period for parasitism before transplanting takes place. Experimentation is now being conducted to improve the timing of treatment of *Trichoderma* to attain practical levels of biocontrol of root-knot control.

4.2 Pathogenicity of *Trichoderma* toward *M. incognita* in soil in the absence of tomato

The results obtained from the interaction between the different biocontrol agents and *M. incognita* in the absence of tomato showed a direct effect of *Trichoderma* and FO162 on *M. incognita*. This was demonstrated as high mortality levels of the nematodes when the sand was treated with the different biocontrol agents. *Trichoderma* isolates were not as effective as FO162. No growth of *Trichoderma* or FO162 on the nematode cuticle or parasitic inside the nematode was detected. Only in one case was *Trichoderma harzianum* (Th3) observed parasitizing the J2. *Trichoderma* may also be more effective egg pathogen and treatment prior to planting when eggs are present and not J2 might gives better results.

4.3 *In vitro* effects of metabolites of *Trichoderma* on mobility and mortality of *M. incognita*

The effect of culture filtrates of the *Trichoderma* on *M. incognita* mobility was clearly demonstrated in the present study. Culture filtrates of *Trichoderma* at concentrations of 50% and 90% caused nematode immobility ranging from 14.3-38% and 11-22 % respectively.

In another study carried by zum Felde (2002) the influence of the metabolites of 10 isolates of *Fusarium* and 9 of *Trichoderma* spp. were screened for nematistatic and nematicidal activity to *R. similis*, and showed little activity and inconsistent nematistatic and nematicidal activity. Her fungi were fermented on Czapek-Dox (CD) media. Media type has been shown to have major effect on production of secondary metabolites (Schuster et al., 1993)

Previous studies carried out by (Amin, 1994; Hallmann & Sikora, 1996) showed that metabolites of endophytic *F. oxysporum* strains decreased significantly the mobility of migratory endo-parasitic nematodes *in vitro*. Metabolites of *F. oxysporum* isolate B20 from a gliotoxin fermentation medium at a concentration of 50% strongly affected *Radopholus similis* mobility (Amin, 1994). Similarly Hallmann and Sikora (1996) showed that the metabolites of *F. oxysporum* strain 162 from a gliotoxin fermentation medium at a concentration of 90% inactivated approximately 65% of J2 and adults of the migratory endoparasitic nematodes *Pratylenchus zeae* and *R. similis*.

The activity of culture filtrates of *F. oxysporum* was 162 also shown to be different for specific trophic groups of nematodes (Hallmann & Sikora, 1996). They showed that after 24 hours exposure plant parasitic nematode species were inactivated between 60–100%, whereas the mobility of mycophagous and bacteriophagous species was not altered. Within the plant parasitic nematode trophic group, sedentary endoparasites were inactivated 100% by metabolites and migratory endoparasite mortality levels reached 65%. Amin (1994) demonstrated mortality of 60–80% by culture filtrate of 2 *F. oxysporum* isolates (A1 and B20) at a concentration of 50% toward *R. similis*. Culture filtrates of *F. oxysporum* were recently demonstrated to inhibit hatching of the sedentary endoparasitic nematode *Heterodera glycines*

and the root-knot nematode *M. incognita* (Meyer et al., 2004) as well as burrowing nematode *R. similis* (Athman et al., 2006).

In the present study, the metabolites of the *Trichoderma* did not differ from FO162 metabolites in level of activity. However, they did significantly decrease the mobility of *M. incognita* when compared to the nematode control. Mortality was lower after rinsing and storing in tap water for 24 hours. After rinsing and storing in tap water for 24 hours, mortality for *Trichoderma* isolates and FO162 ranged from 14-39% as compared to 3-10% in controls.

In previous studies, mortality of root-knot nematode was associated with the disintegration of internal tissue of the nematodes tested caused by culture filtrates of mutualistic endophytic *F. oxysporum* (Amin, 1994; Schuster et al., 1995; Hallmann & Sikora, 1996). Second stage juveniles of the migratory endoparasitic nematode *Radopholus similis* was more sensitive to fungal metabolites than older juvenile stages or adults Amin (1994).

5. Conclusion

From the previous results the following can be concluded:

1. Simultaneous inoculation of *Trichoderma* and root-knot nematode one week before transplanting resulted in higher level of control than simultaneous inoculation at transplanting in both numbers of galls and number of egg masses. The level of reduction was not satisfactory due to the short interval of exposure of the nematode to the antagonist.

2. Inoculation of the infested soil with the fungi at least 3 weeks before transplanting is probably needed to obtained adequate levels of control with *Trichoderma*.

3. In the present tests isolates of *Trichoderma* did not give levels of control equal or above FO162 and were distinctly inferior to the endophyte.

4. No clear positive or negative effects of *Trichoderma* on plant growth were detected throughout the study.

5. *Trichoderma* reduced *M. incognita* survival in soil in the absence of tomato plants, which indicated direct toxicity to eggs or J2.

6. *Trichoderma* also produce toxins in culture filtrates that affect mobility of *M. incognita in vitro*. This further strengthens the hypothesis that direct toxic effects on the nematode in the soil exist. There is a need for extended exposure times of *Trichoderma* to root-knot nematode before transplanting for adequate control.

Chapter Four

IMPORTANCE OF APPLICATION TIME AND INOCULUM DENSITY OF THE NON-PATHOGENIC ENDOPHYTIC FUNGUS *FUSARIUM OXYSPORUM* 162 FOR THE BIOLOGICAL CONTROL OF THE ROOT-KNOT NEMATODE *MELOIDOGYNE INCOGNITA* ON TOMATO

1. Introduction

Many different microorganisms have been used for the biological management of nematodes in soils. Bacterial and fungal agents have been used effectively to reduce a range of plant parasitic nematodes (Kerry, 2000; Hallmann et al., 2001; Meyer and Roberts, 2002). The mechanisms of antagonism vary greatly and include predation, pathogenesis, competition, repellence and induced resistance (Jaffee and Zehr, 1985; Stirling, 1991; Timper et al., 2005). Interest has recently shifted to bacterial and fungal antagonists that reside endophytically in the endorhiza of the host plant (Pocasangre et al., 2000; Sikora et al., 2003). Endophytic microorganisms can easily be applied to seeds or transplants, thereby reducing the amount of inoculum and overall costs of nematode management compared with soil application (Harman, 1991; Sikora et al., 2000; Elzein et al., 2006).

Many fungi have been shown to grow endophytically in the endorhiza (Hussey and Roncadori, 1982; Sikora, 1992; Waceke et al., 2001; Sikora et al., 2003). One of the most predominant non-pathogenic fungus found in roots of many plant species is *Fusarium oxysporum* (Alabouvette et al., 1998) with the exception of forma specialis of *F. oxysporum* that are important pathogens on many crops (Alabouvette et al., 1998; Larkin and Fravel, 1999). The vast majority of these *F. oxysporum* strains however, are non-pathogenic saprophytes facultatively colonizing the root (Sikora, 1992). Non-pathogenic strains of *F. oxysporum* have been used effectively for the control of fungal plant pathogens (Kroon et al., 1991; Lemanceau and Alabouvette, 1991; Alabouvette et al., 1993) and are used in the field to control *Fusarium* wilt of tomato (Alabouvette et al., 1993).

Non-pathogenic strains of *F. oxysporum* also have been shown to reduce root-knot nematode infection of tomato (Hallmann and Sikora, 1994a; 1994b; Sikora et al., 2003). For example, inoculation of tomato seedlings with the 162 strain of *F. oxysporum* reduced gall formation of tomato caused by *M. incognita* up to 75% (Sikora et al., 2003). In addition, root growth was significantly enhanced.

Non-pathogenic strains of *F. oxysporum* have also been shown to reduce population densities of lesion nematodes on maize (Kimenju et al., 1998) and burrowing nematodes on banana (Niere et al., 1998, 1999; Pocasangre, 2000; zum Felde et al., 2005). Banana tissue culture transplants reared in substrate pre-inoculated with non-pathogenic *F. oxysporum* and challenged 5 weeks later with *Radopholus similis* had 50% lower nematode densities in their root compared with the untreated control plants (Sikora et al., 2000; Vu, 2005). Sikora and Pocasangre (2004) obtained a 86% reduction of *R. similis* densities in plantain suckers following inoculation with non-pathogenic *F. oxysporum.* Control of *R. similis* under greenhouse and field conditions has been reported by several authors (Schuster et al., 1995; Niere et al., 1998; zum Felde et al., 2005).

Control efficacy is often inconsistent due to both abiotic and biotic factors affecting antagonist's colonization and growth in the endorhiza. The most important factors affecting control are inoculum production and improper application technology. It has been demonstrated for example that control efficiency toward the wilt pathogen *F. oxysporum* f. sp. *lycopersici* on tomato following pre-inoculation with non-pathogenic *F. oxysporum* strains depends on the ratio of these two fungi in the soil (Alabouvette et al., 1993; Minuto et al., 1995; Duijff et al., 1999). Therefore, determination of optimum inoculum densities leads to more effective endophytic colonization of the roots and thereby to a higher level of control. Bao and Lazarovits, (2001) further showed that the ratio of propagules of the non-pathogenic *F. oxysporum* strain 70T01 to those of the *Fusarium* wilt pathogen need to be equal favouring the non-pathogenic strain to lower the population of pathogenic *F. oxysporum* f. sp. *lycopersici* .

Studies on the optimum inoculum density of non-pathogenic *F. oxysporum* required for the management of nematodes or pathogens on vegetables are still lacking. Most researchers used only one inoculum density: 2.75 x 10^7 cfu/seed for the control of *M. incognita* on tomato (Hallmann and Sikora, 1994b), 10^7 conidia/ml to control *F. oxysporum* f. sp. *lycopersici* on tomato (Kroon et al., 1991) and 1.2 x 10^6 cfu/ml to control *Radopholus similis* on banana (Pocasangre et al., 2000). Mandeel and Baker (1991) used two non-pathogenic strains of *F. oxysporum* at levels of 1.08 x 10^4 and 5 x 10^4 cfu/g soil to control *Fusarium* wilt of cucumber. In cross-protection studies Alabouvette et al. (1993) used 10^6 cfu/ml soil to control *Fusarium* wilt on tomato. In addition, the methods of application vary greatly: surface drenching of the seedlings, soil incorporation, and root ball dipping (Hallmann and Sikora, 1994a; Olivain and Alabouvette, 1999; Paparu et al., 2004)

In previous studies with FO162 on tomato the level of control varied significantly. A principal factor for this variation is poor colonization of the biocontrol agent into the root which is mostly determined by inoculum densities and time of inoculation, since all other factors are stable. For this reason the following experiments were designed to determine the:

1. Most efficient method of application.

2. Optimum inoculum density for effective root colonization

3. Relationships between inoculation density, root colonization and control.

4. Influence of FO162 on long term control

2. Materials and Methods

2.1 Application time on biocontrol

Tomato seeds were sown at one seed/plug into commercial 70 plug seedling trays, with each plug measuring 4x4.5x2.5 cm. One of the two trays was treated with FO162 and the other was left untreated. Treatment was made one week after sowing. Each plug of the treated tray was drenched with 1 ml of a liquid suspension of FO162 containing 1.5 x 10^7 spores ml^{-1}. The fungal inoculum was inoculated into the rhizosphere through 3 holes made with a plastic rod around the seedling stem base. Plugs of the untreated tray were treated with tap water. Seedlings were kept for 5 weeks in the greenhouse and then transplanted into pots (11 x 14 x 9.5cm) containing 800 g of a sterilized sand:soil mixture (2:1, v/v), respectively. Immediately after transplanting, 3 ml of tap water containing 1000 second-stage juveniles of *M. incognita* were inoculated into the pots where nematode inoculation was required (see treatment details below). The nematode inoculum was applied into 3 holes around the plant. FO162 was also applied in one ml of tap water consisting 1.5 x 10^7 cfu per plant to the designated treatment at transplanting.

The experiment consisted of the following five treatments: 1) untreated control, 2) *M. incognita* at transplanting, 3) FO162 at sowing, 4) FO162 at sowing + *M. incognita* at transplanting, and 5) FO162 at sowing and at transplanting + *M. incognita* at transplanting. The pots were placed in a greenhouse at 22ºC ±5 and a16-hour photoperiod.

The experiment was terminated eight weeks after nematode inoculation, i.e. 13 weeks after fungal inoculation at sowing. The roots were removed and washed free of soil. Root and shoot fresh weight as well as plant height were measured. Roots were then stained with 0.015% Phloxine B for 20 minutes, rewashed again with tap water to remove extra stain and then the number of galls and egg masses was recorded.

The level of FO162 endophytic colonization was determined. The roots were surface sterilized by soaking them in a solution of 1.5% NaOCl for three minutes, followed by three rinses in sterilized water. Approximately 12 sections each of 0.5 cm length were cut from similar

diameter roots and placed onto PDA. Successful re-isolation was confirmed when the growth characteristics of the outgrowing fungi corresponded with FO162.

2.2 Application time and inoculum density on biocontrol

In this experiment the treatments used in 2.1 on application time were expanded by adding 2 inoculum densities. Tomato seeds were sown at one seed/plug into commercial 70 plug seedling trays. One of the two trays was treated with FO162 as described in the previous experiment and the other was left untreated. One week after sowing, each plug of the treated tray was drenched with 1 ml of a liquid suspension of FO162 containing either 10^4 or 10^5 spores per gram seedling substrate. The fungal solution was inoculated into the rhizosphere through 3 holes made with plastic rod around the stem base. Plugs of the untreated tray were treated with tap water. Seedlings were kept for 5 weeks in the greenhouse and then transplanted into pots (7x7x8 cm) containing 400 g of a sterilized sand:soil mixture (2:1, v/v), respectively. One day after transplanting all pots were inoculated with 3 ml tap water containing 1000 second-stage juveniles of *M. incognita.* The inoculum was applied into 3 holes around the plant as described for *F. oxysporum*. At this time, specific treatments received an additional dose of FO162 by applying 10^4 or 10^5 cfu per gram soil in 3 holes made into the rhizosphere of the tomato plants.

Two weeks after nematode inoculation the roots were washed free of soil with tap water and root weights were recorded. The roots were then stained with Fuchsin acid by heating in the microwave for 1.5-2 minutes. They were then washed with water and cut into 1cm lengths and macerated with an Ultra Turrax® T25 in water. The roots of individual plants were then poured into a 100 ml graduated cylinder and filled up to 100 ml with tap water. The cylinder was agitated and then a 10 ml aliquot removed for nematode counting. The experiment consisted of 10 treatments each was replicated 10 times (table 4.1).

Table 4.1 List of treatments

Number	Treatment		*M. incognita*
	FO162 cfu/g seedling substrate at sowing	FO162 cfu/g soil at transplanting	
1	10^4	0	+
2	10^4	10^4	+
3	10^4	10^5	+
4	10^5	0	+
5	10^5	10^4	+
6	10^5	10^5	+
7	0	10^5	+
8	0	10^4	+
9	0	0	+
10	-	-	-

2.3 Influence of *F. oxysporum* 162 on *M. incognita* fecundity

This experiment followed the same procedures as mentioned above. Fungal inoculation was 10^4 or 10^5 cfu/g seedling substrate and/or 10^4 or 10^5 cfu/g soil at transplanting. Four treatments were used: 1) Absolute control, 2) *M. incognita*, 3) *M. incognita* + FO162 at sowing, 4) *M. incognita* + FO162 at sowing and transplanting. Each of these treatments was replicated 10 times. Eight weeks after nematode inoculation the plants were uprooted and the roots were gently washed and weighed. The number of galls and egg masses were then recorded (see section 2.1 in this chapter). These roots were used again to extract the eggs from the egg masses as described by Hussy and Barker, (1973).

3. Results

3.1 Effect of application time

There were no significant differences in the overall level of control attained between the two treatment times in both experiments. Treatment at sowing alone as well as dual inoculation at sowing and at transplanting led to similar significant reductions in the number of galls and egg masses per plant. Multiple inoculations gave a slightly higher reduction in the number of egg masses and galls than the single treatment at sowing (figure 4.1). However, these differences were not significant from one another.

Similarly in the second experiment, gall numbers were significantly reduced by the dual inoculation treatment at sowing and transplanting as well as by single treatment at sowing compared to the control (figure 4.2). However, these differences were significant from one another.

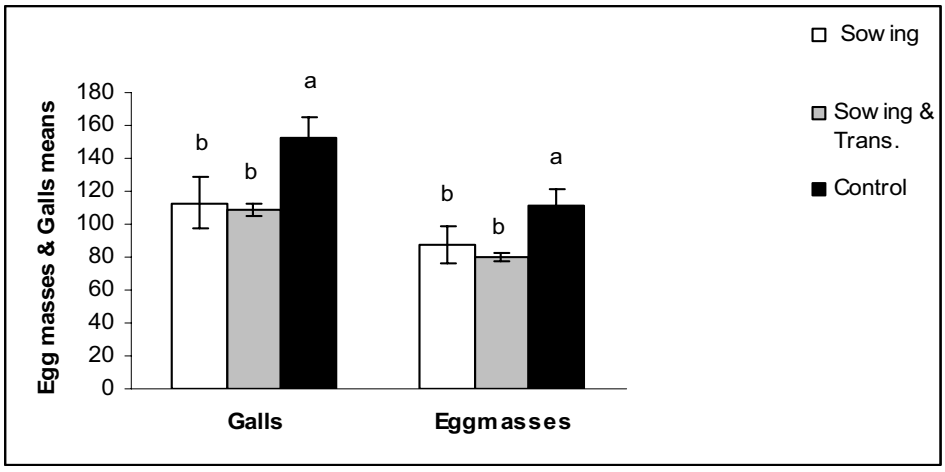


Figure 4.1 Effect of *Fusarium oxysporum* 162 application time on the number of galls and egg masses formed by *Meloidogyne incognita* on tomato. Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

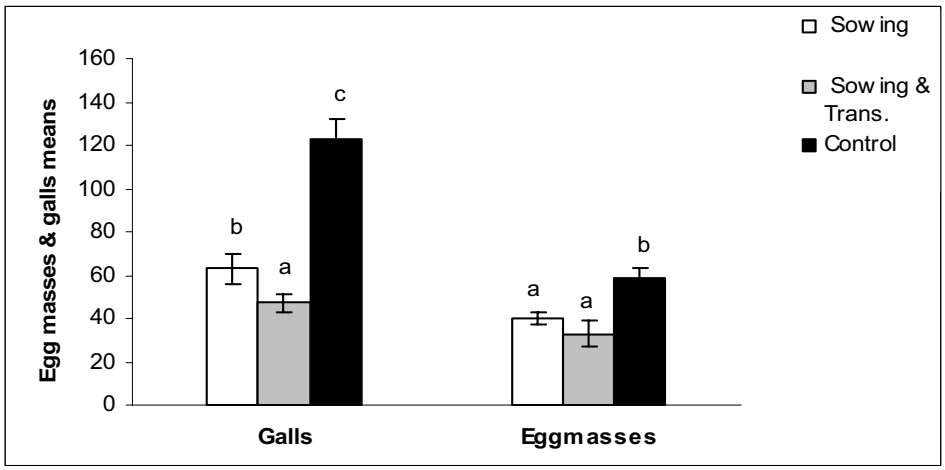


Figure 4.2 Effect of *Fusarium oxysporum* 162 application times on the number of galls and egg masses formed by *Meloidogyne incognita* on tomato. Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

3.2 Application time and inoculum density on biocontrol

Treatment with either dose of FO162 at sowing led to a significant reduction of up to 75% in nematode penetration in both tests (table 4.2 & 4.3). Treatment only at transplanting caused a significant reduction in nematode penetration in the first test but not in the second.

Multiple treatments of the plants at sowing and at transplanting at both dose levels gave significantly higher levels of control when compared to the control. However, there were no significant differences between the doses used in the combination treatments (table 4.2). In the second experiment, the level of control was more variable which led to a lack of significant levels of control in some treatments.

Multiple inoculations of the seedlings with 10^5 cfu/g of FO162 of seedling substrate at sowing followed by 10^4 cfu/g soil at transplanting resulted in the highest reductions in nematode infection over the controls in both tests (table 4.2 & 4.3).

Table 4.2 Effect of *Fusarium oxysporum* 162 inoculation time and density on *Meloidogyne incognita* penetration per gram root of tomato plants 2 weeks after nematode inoculation.

Treatments		Means
FO162 cfu/g seedling substrate at sowing	FO162 cfu/g soil at transplanting	Nematode penetration
Plant alone	Plant alone	--
10^4	0	49 ab[1]
10^5	0	48 a
10^4	10^4	40 a
10^5	10^5	46 a
10^4	10^5	44 a
10^5	10^4	31 a
0	10^5	80 bc
0	10^4	90 c
0	0	125 d

[1] Values with different letters in the same column are significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

Table 4.3 Effect of *Fusarium oxysporum* 162 inoculation time and density on *Meloidogyne incognita* penetration per gram root of tomato plants 2 weeks after nematode inoculation.

Treatments		Means
FO162 cfu/g seedling substrate at sowing	FO162 cfu/g soil at transplanting	Nematode penetration
Plant alone	Plant alone	--
10^4	0	232 ab[1]
10^5	0	258 a-c
10^4	10^4	281 b-d
10^5	10^5	325 c-e
10^4	10^5	294 b-e
10^5	10^4	190 a
0	10^5	307 b-e
0	10^4	355 de
0	0	366 e

[1]Values with the different letters in the same column are significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

3.3. Influence of FO162 on *M. incognita* fecundity

Seedlings inoculated with FO162 significantly reduced initial nematode damage when application was done at sowing or at sowing and transplanting (figure 4.3). Dual inoculation

gave slightly higher nematode control, but it was not significantly different to single inoculation at sowing.

Treatment at sowing alone as well as double inoculation of the fungus at sowing and at transplanting led to similar significant reductions in the number of galls and egg masses per plant.

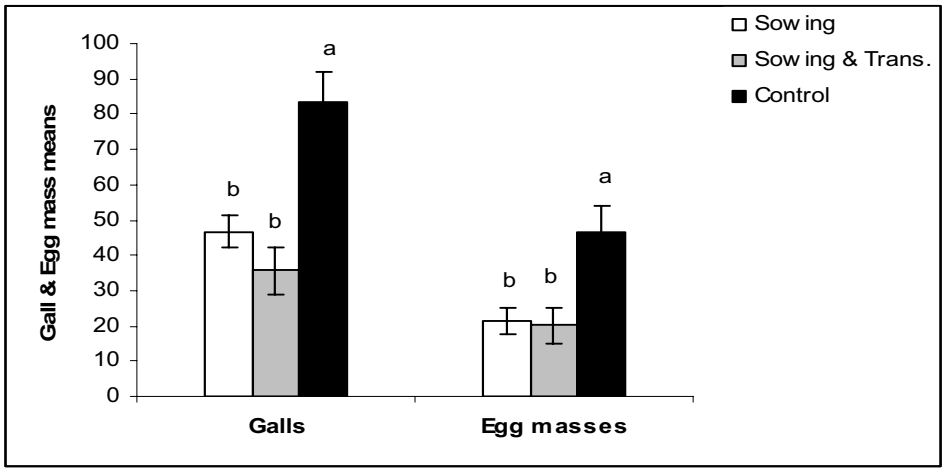


Figure 4.3 Influence of *Fusarium oxysporum* 162 inoculation at sowing and at sowing and transplanting on *Meloidogyne incognita* galling and number of egg masses. Means with the same letter are not significant difference based on Tukey Test ($P \leq 0.05$; n=10)

Whether multiple inoculations have an effect on the second generation of *M. incognita* and on the number of eggs per egg mass also was determined. Figure 4.4, shows that there were no differences in the number of eggs per egg mass between the different treatments. This is an indication that neither female growth nor fecundity is affected following FO162 treatment.

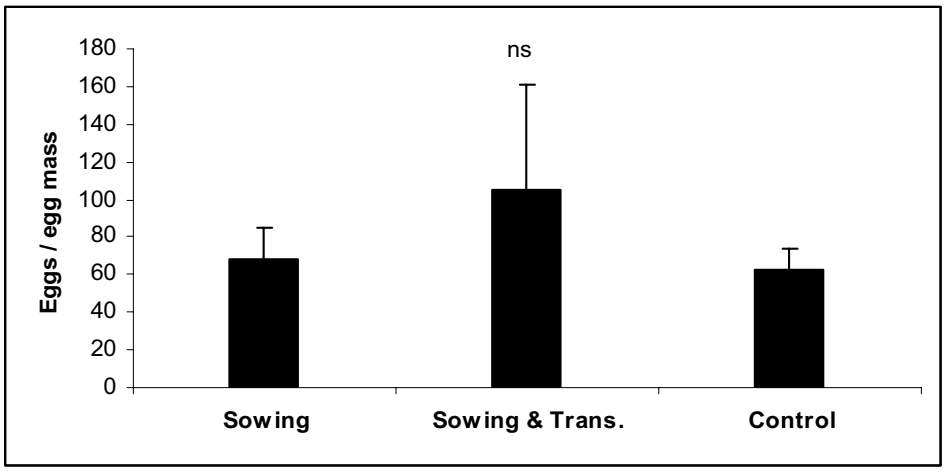


Figure 4.4 Influence of *Fusarium oxysporum* 162 inoculated at sowing and at sowing and transplanting on *Meloidogyne incognita* eggs per egg mass. Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n=10)

However, multiple inoculations with FO162 caused a significant reduction in egg number per root system which reflected initial effects on root-knot nematode penetration as measured in gall number (figure 4.5)

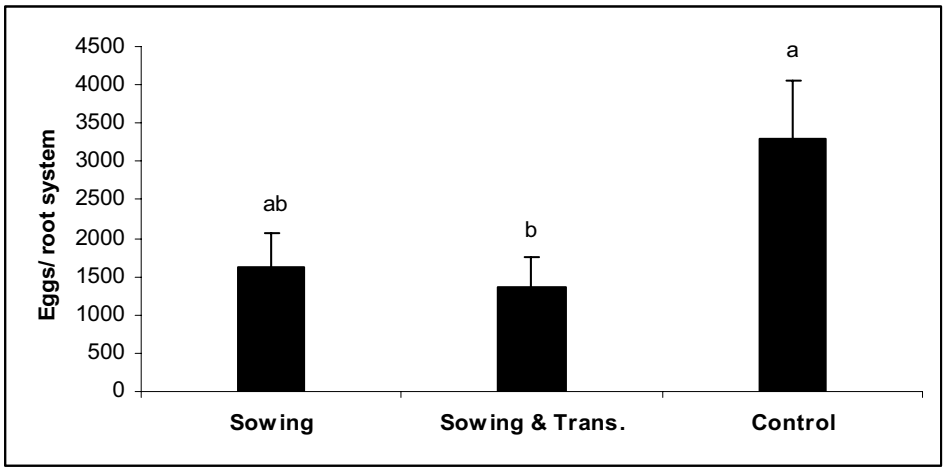


Figure 4.5 Influence of *Fusarium oxysporum* 162 inoculated at sowing and at sowing and transplanting on *Meloidogyne incognita* eggs per root system. Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n=10)

No significant effect of the endophytic fungus on the root weight was detected in this experiment when inoculated at sowing or at sowing and transplanting stages (figure 4.6)

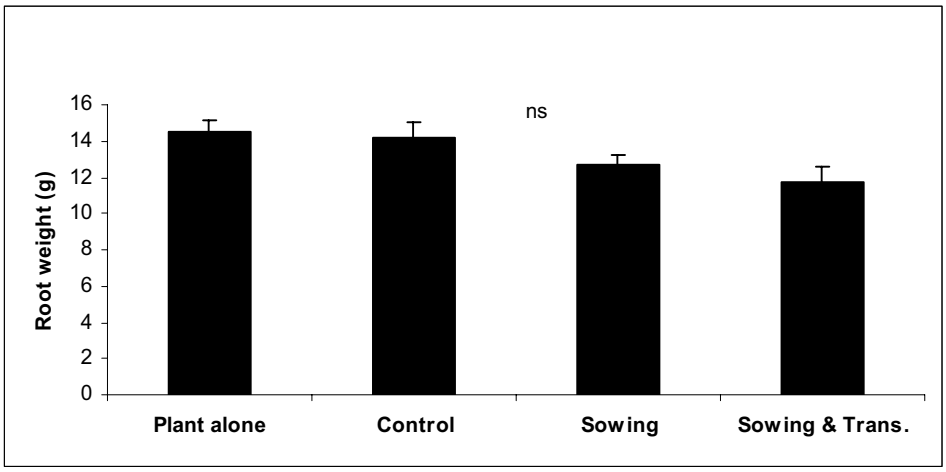


Figure 4.6 Influence of *Fusarium oxysporum162* inoculation at sowing and at sowing and transplanting on root weight. NS: Means are not significantly different based on Tukey Test ($P \leq 0.05$; n=10).

4. Discussion

4.1 Inoculation time

The results of the tests on the importance of inoculation time on the level of biocontrol showed that *M. incognita* infection measured as the number of galls per plant was significantly reduced when FO162 was applied at sowing or at sowing and again at transplanting. Multiple treatment of plants generally led to slightly higher levels of control though not always significant when compared to single application. Hallmann and Sikora (1994a, b), Sankaranarayanan et al. (2002) and Diedhiou et al. (2003) showed that strains of non-pathogenic *F. oxysporum* reduced root-knot nematode infestation by preventing juveniles from invading the roots and by interfering with juvenile development within the root tissue.

The effect on egg mass production, which is a measure of female development over time, was significantly reduced in both experiments by both application forms. The reduction in the number of egg masses was probably caused by a delay in penetration of the root due to repellent activity of FO162 on the juveniles observed in other experiments (see chapter 5).

Neither shoot and root weights nor plant heights varied between treatments (data not shown). The results obtained are similar to those of Hallmann and Sikora (1994a) who reported that shoot and root fresh weights were not significantly affected by FO162 treatment. The same lack of growth response was reported for plant height by Sankaranarayanan et al. (2002).

4.2 Application time and inoculum density

In the experiments on the interrelationship between application times with inoculum density, inoculation with FO162 at sowing caused a significant reduction in penetration at both inoculum levels. The level of reduction was not improved significantly by increasing the dose from 10^4 to 10^5. The higher level of nematode penetration in the second experiment was attributed to higher temperatures in the greenhouse during the summer (28ºC ± 5) compared with the first test which was carried out in winter at cooler temperatures (18ºC ±2).

Inoculation at transplanting gave lower levels of nematode control when compared to treatment at sowing. This was most likely caused by low levels of endophyte colonization in the short period between inoculation and nematode introduction. Meyer and Roberts (2002) stated that biocontrol agents must be applied in combination to increase colonization of the rhizosphere soil in order to control plant parasitic nematodes and soilborne plant-pathogenic fungi. The level of microbial activity in the rhizosphere of tomato needed for suppression of *Fusarium* causing wilt diseases is density dependent. The ratio of non-pathogenic *F. oxysporum* Fo47 to pathogenic *F. oxysporum* f. sp. *lini* strain Foln3GUS must be 100:1 for suppression to be significant (Duijff et al., 1999).

Dual inoculation did not significantly increase the overall level of nematode control over that attained with a single inoculation of FO162 at sowing. This demonstrated that the level of endophyte colonization required for control is already obtained when the antagonist is introduced 5 weeks before exposure to the nematode. Treatment at transplanting, which does not allow for good colonization or control, adds to overall costs of the treatment and therefore is not recommended.

Further optimization of inoculum production and improvement in formulation of FO162 could improve control and reduce the costs for nematode management. For example, Elzein et al. (2006) suggested that coating the seeds with *Fusarium oxysporum* for control of the root parasitic weed Striga, appears to be an attractive option for minimizing the amount of inoculum required. Finding isolates of *F. oxysporum* that colonize quickly and at even lower inoculum levels could reduce treatment costs.

Tomato seed treatment with such isolates would establish the biocontrol agent in the potential colonization zone of the young seedling, and offer a simple, easy and economical delivery system for large scale seedling production nurseries.

4.3 *M. incognita* fecundity

Whether inoculation of tomato with FO162 will enhance control effectiveness in succeeding nematode generations is important. If fungal colonization of the roots decreases over time, it

could lead to uninhibited reproduction of the nematode in later generations and damage at the end of the growing season (see chapter 6).

The study to determine if single or multiple inoculations has an effect on the population dynamics of root-knot nematode over time was done using eggs per egg mass as a factor. The results showed no differences in the number of eggs per egg mass, indicating no effect on root-knot nematode female development and fecundity. The drop in egg per root system was a reflection of the reduction in penetration and therefore the number of egg laying females. FO162 therefore has its main biocontrol activity only on penetration.

The results of these experiments demonstrated very clearly that the optimum time for introduction of the mutualistic endophytic fungal antagonist FO162 was at the time of sowing for production of seedlings. In addition the results showed that inoculum densities as low as 10^4, when applied at sowing, were sufficient to give high levels of biocontrol after transplanting into root-knot nematode infested soil due to good colonization of the root system with FO162. The level of control was not improved significantly when higher inoculum densities were used. The results are of importance for field application in that a stable, simple and economically adaptable nematode control system can be achieved through biological enhancement of tomato with endophytes in seedling production systems.

It should be noticed that initial inoculum levels for seedling bio-enhancement with FO162 or other endophytes isolates might be dependent on the tomato variety. The effect of variety, for example, varieties resistant to *Fusarium* wilt need more detailed study. Differences between varieties were investigated in (chapter 6).

5. Conclusion

The biocontrol activity of the mutualistic endophytic fungus FO162 against *M. incognita* was tested under different inoculum densities and times of inoculation. The results of this study led to the following conclusions:

1. A single application of the endophytic fungus FO162 at sowing was sufficient to reduced nematode damage.

2. The level of reduction was not improved significantly by increasing the dose from 10^4 to 10^5 therefore 10^4 seems to be optimum for this variety.

3. Eggs per egg masse were not significantly affected by treating with FO162, indicating no effect on root-knot nematode fecundity.

4. FO162 was not pathogenic when root weights were recorded.

Chapter Five

MECHNISIMS OF ACTION ASSOCIATED WITH THE MUTUALISTIC ENDOPHYTE *FUSARIUM OXYSPORUM* 162 TOWARDS ROOT-KNOT NEMATODE *MELOIDOGYNE INCOGNITA* ON TOMATO

1. Introduction

One of the most important aspects of biological control is the study of the mechanisms employed by biocontrol agents to reduce diseases. To be able to investigate this interrelationship it is important to understand the biology of the pest or disease and the infection process and then find weak links in the life cycle that favor biocontrol.

Soilborne and endophytic fungi have been shown to be an active part of the naturally occurring antagonists of nematodes and have the potential to regulate plant parasitic nematode populations. Biocontrol agents act against plant pathogenic nematodes through different mechanisms of action that are not necessarily plant based. These antagonistic interactions lead to biocontrol by: predation (Stirling, 1991), parasitism (Kerry, 2000), pathogenesis (Schuster et al., 1995), competition (Sikora, 1992; Diedhiou et al., 2003; Fravel et al., 2003; Sikora et al., 2003), repellence (Vu, 2005) and induced resistance (Hasky-Günther and Sikora, 1995; Reitz et al., 2001; Reitz and Sikora, 2001).

Plant based mechanisms against pathogens are based on a variety of constitutive barriers already present in the plant before infection. The combined effect of these barriers is referred to as constitutive resistance. Plants can also activate protective mechanisms upon contact with invaders; and this is termed induced or acquired resistance (Kessmann et al., 1994; Sticher et al., 1997).

The mechanisms of action involved in cross-protection against *Fusarium* wilt on tomato and cucumber by non-pathogenic *F. oxysporum* has been well investigated (Biles and Martyn, 1989; Mandeel and Baker, 1991; Fuchs et al., 1997). They were shown to be able to compete for nutrients in the soil, affecting the rate of chlamydospore germination of the pathogen.

They also competed for infections site on the root, and can trigger plant defence reactions, inducing systemic resistance (Fravel et al., 2003).

Induced resistance has been considered an important mechanism responsible for disease control (Fuchs et al., 1997; Alabouvette et al., 1998). Although, bacterial induced systemic resistance has been demonstrated as a mode of action for biocontrol activity against cyst nematodes (Hasky-Günther et al., 1998; Reitz and Sikora, 2001; Reitz et al., 2002) and toward root-knot nematode (Siddiqui and Shaukat, 2004; Schäfer et al., 2005). Nothing is known about the mechanisms involved in the biocontrol of root-knot nematode by *F. oxysporum*.

Hallmann and Sikora (1994a, b) who used many strains of *F. oxysporum* to control root-knot nematode on tomato, demonstarated that *F. oxysporum* is cabable of reducing both number of galls and eggmasses compared with the control plants. They attributed the reduction in nematode infection to possible fungal toxins or to competition of the fungi for space with the parasite (Hallmann and Sikora, 1996).

Competition in the rhizosphere and at infection sites as well as induction of enhanced resistance in the host when nonpathogenic *Fusarium oxysporum* were used against *Fusarium* wilt on cucumber was confirmed by Mandeel and Baker (1991) as the factors responsible for the mechanisms of wilt suppression.

Despite the high levels of control attained by the mutualistic endophyte *F. oxysporum* 162 in controlling root-knot nematodes on different crops, its mode of action is still not well investigated. The objectives of the following research on the mechanisms of action involved in reducing root-knot nematode infection were:

1- The influence of the FO162 on nematode development in the root.

2- The activity of the FO162 on root-knot nematode attraction and penetration.

3- Whether FO162 induces systemic resistance.

4- The presence of repellent activity of FO162.

2. Materials and Methods

2.1 Development in tomato roots

In this experiment J2 penetration was manipulated so as to allow only 48 hours for penetration. This resulted in synchronized development of the females in both treated and untreated plants. Tomato seeds were sown at one seed/plug into 35 plug seedling trays, with each plug measuring 6x6x4cm. One of two trays was treated with FO162 and the other was left untreated. One week after sowing, each plug of the treated tray received 5 ml of a liquid suspension of FO162 containing 1x 10^6 spores g^{-1} sand. The fungal suspension was inoculated into the rhizosphere through 5 holes made with a plastic rod around the stem base. Plugs of the untreated tray were treated with tap water and served as control. Seedlings were kept for 5 weeks in the greenhouse and then inoculated with 3 ml tap water containing 1000 second-stage juveniles of *M. incognita.* The inoculum was applied into 3 holes around the plant as described for *F. oxysporum.* After 48 hours the plants were removed from the plugs and the roots were carefully washed with tap water to free them of sand and non-penetrated nematodes. They were then replanted into new pots (7x7x8 cm) containing 400 g of sterilized sand:soil mixture (2:1, v/v), respectively. Each treatment was replicated 6 times.

The plants were incubated in the greenhouse for 23 days and then the test was terminated. The roots were removed and washed free of soil. The roots were then stained with Fuchsine acid as described in chapter 4 and the number of different nematode growth stages (figure 5.5) was counted under the microscope.

2.2 Juvenile attraction and penetration

Tomato seeds were sown in seedling trays. The plugs were 6x6x4 cm with 35 plugs per tray. One week after sowing, one tray was inoculated with 1x 10^5 spores of FO162 g^{-1} seedling substrate, and the other tray left untreated and used as a control.

After six weeks, the tomato seedlings were transplanted into a Linked Twin-pot Chamber. Each pot measured 9x9x8 cm, and was filled with 650 cm^3 of a sterilized sand:soil mixture (2:1, v/v), respectively. The two pots were connected by a plastic bridge filled with the same soil substrate (figure 5.1). The 3-sided bridge was constructed out of plastic 1x1.2 x4 cm. Two

days after transplanting, 1000 second-stage juveniles were inoculated at the middle of the bridge. The bridge was covered with a plastic lid to maintain moisture.

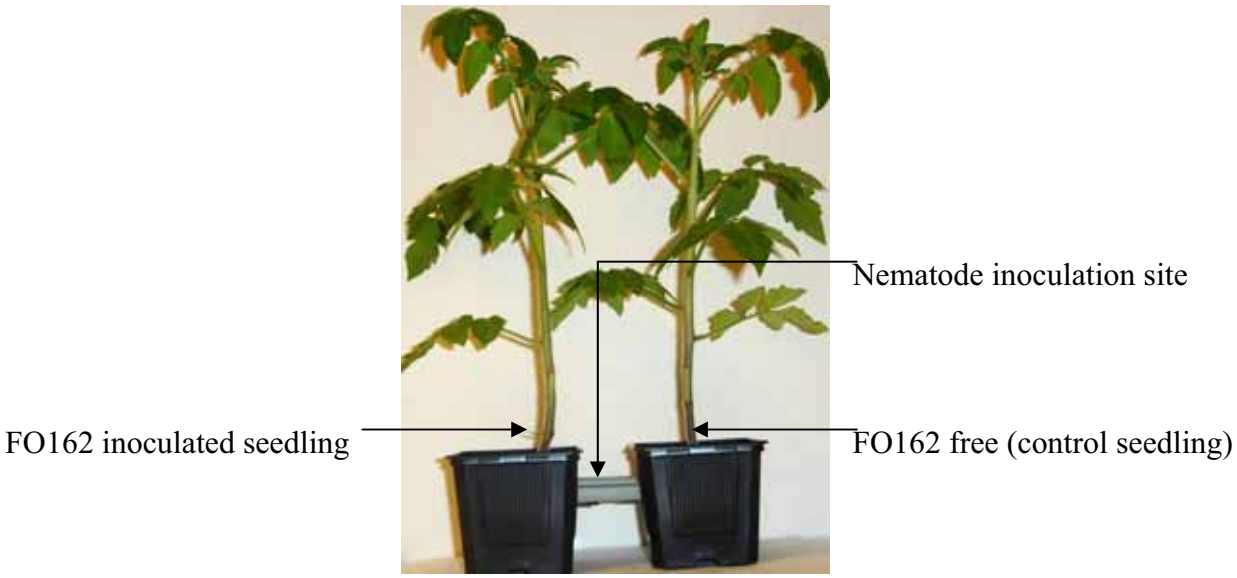


Figure 5.1 Design of Linked Twin-pot Chamber used to determine the influence of *Fusarium oxysporum* 162 on *Meloidogyne incognita* attraction and penetration.

Two experiments were carried out to examine nematode migration behaviour. The first experiment consisted of two treatments: 1) fungus free seedlings on both sides, and 2) one seedling treated with fungus and the other left untreated.

In the second experiment an additional treatment was used with both seedlings inoculated with the fungus.

Two weeks after nematode inoculation, the roots from each pot were freed of soil by washing them with tap water. The roots were then weighed. The roots were stained with an acid fuchsin-lactoglycerol solution, cut into ca 2 cm long pieces and then homogenized in a blender (Turax) for approximately 30 seconds. The macerated root solution was diluted to 100 ml with tap water and 10 ml taken for nematode counting under the binocular microscope.

2.3 Induced systemic resistance

Tomato seeds were sown in seedling plugs as outlined in (2.1). After three weeks the seedling plugs were transplanted to individual 10 cm Φ plastic pots until they reached a height of 25 to 30 cm. At this stage the shoot was detached from the root system. The basal part of the shoot was split into 2 halves (5 cm in length) and each half was placed in a separate pot. The pots were filled with 600 g of a sand:soil mixture (2:1, v/v), respectively. One week after planting the newly formed roots on one half were treated with one ml of a liquid suspension of FO162 adjusted to $1x10^5$ cfu g^{-1} soil. This side was termed as the Inducer side (I). The inoculum was injected into the rhizosphere using 3 holes made around the stem base with a plastic rod. The other half of the root acted as the control and was treated with tap water. One week after FO162 inoculation, the other side of the root was inoculated with a 3 ml solution containing of 1000 second-stage juveniles of *M. incognita* by injecting the solution into 3 holes roughly 2 cm deep around the stem base. This side was termed as the Responder (R), (figure 5.2).

The experiment consisted of two treatments: 1) Inducer side treated with water and Responder side inoculated with *M. incognita*, and 2) Inducer side inoculated with FO162 and the Responder side inoculated with *M. incognita.* Each treatment was replicated 8 times. The experiment was terminated at two stages: 1) after 2 weeks to investigate whether treatment affected early root penetration, and 2) after 5 weeks to study the influence of the biocontrol agent on nematode galls and egg mass production.

Penetration was measured by washing both root halves with tap water to free them from soil. They were then stained separately with Fuchsine acid by heating in a microwave for 1.5-2 minutes. One hour later, they were washed with water to free from stain and then macerated with an Ultra Turrax® T25 for 1 minute. The macerate was then diluted to 100 ml with water and mixed thoroughly by shaking. Counting chambers were then filled with 10 ml of the macerated root solution and nematode penetration was determined under a binocular microscope.

The number of galls and egg masses of the nematode were determined after washing gently and weighing the roots in each pot half. The roots were then stained with 0.015% Phloxine B

for 20 minutes and washed with tap water to free them from excess stain. The number of galls and the number of egg masses on each root-half was counted.

Figure 5.2 Design of Split-Root Chamber used to determine *Fusarium oxysporum* 162 induced systemic resistance against *Meloidogyne incognita* in tomato.

2.4 Repellent activity of root exudates

Tomato seeds were sown in pots (9x9x8 cm) filled with 650 g of a sterilized sand. One week after sowing, 6 pots were treated with FO162. The soil was treated by adding 5 ml of a liquid suspension of FO162 adjusted to 1x10^5 cfu g^{-1} soil. The inoculum was injected into the rhizosphere using 3 holes made around the stem base with a plastic rod. Pots of the untreated plants were treated with tap water. Seedlings were maintained for 5 weeks in the greenhouse after which the plants were left without water for 2 days or just before reaching the permanent wilting stage. Each pot was then flooded with 200 ml of water and allowed to stand for percolation of the water through the pot-holes into the pot bottom. The exudate was collected together in separated beakers for all control plants and for FO162 treated plants. The total amount of root washing per treatment was about 600 ml for all plants. The two exudates

solutions were filtered by pouring them into other beakers through a filter paper (Schleicher & Schuell MicroScience-Germany) to remove debris before being used in the experiment.

Elongated chambers 10x2x2 cm made of plastic were filled with moisturized sterilized fine sand 100-200 µm sieve aperture. The chambers were placed in the greenhouse and treated as follows by pipetting 3 ml of the respective solution at the far side of each arm: 1) both arms inoculated with FO162-root exudate, 2) both arms inoculated with root exudate, 3) one arm inoculated with FO162 root-exudate and the other arm with control root exudate, and 4) both arms treated with distilled water as the absolute control. Immediately thereafter, 1000 J2 of *M. incognita* were inoculated in the middle of each chamber, and the chamber covered with plastic foil to reduce the evaporation rate. The chambers were retreated at 24 hours intervals with 1 ml of the same treatment solutions to keep the chamber moisturized. One week later, the sand in each chamber was divided into equal halves and each half collected in 50 ml test tubes and then filled with tap water. A 2 ml aliquot of each test tube solution was taken after thorough shaking and the nematodes were counted.

This experiment was repeated 2 times. The same procedure as mentioned above was followed in the 2nd experiment with the exception that 1500 J2 were inoculated in the second test. Each treatment was replicated 5 times.

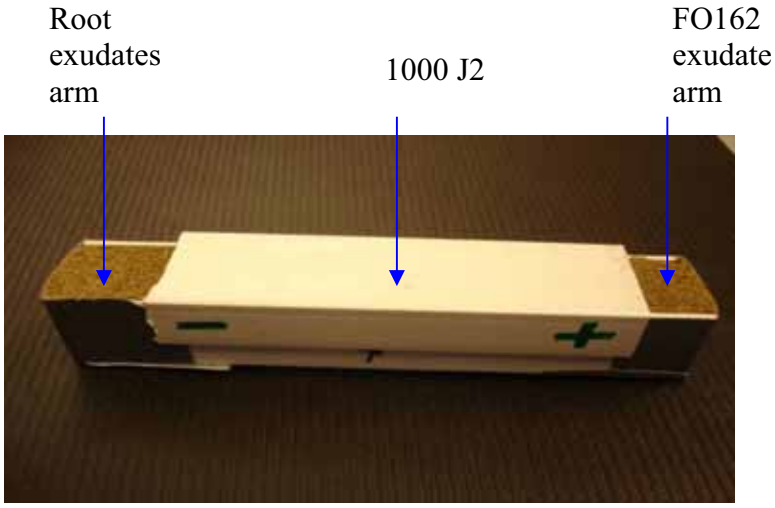


Figure 5.3 Design of the chamber used to determine the influence of *Fusarium oxysporum* 162 root exudate on *Meloidogyne incognita* movement in soil.

3. Results

3.1 Development in tomato roots

In this experiment with synchronized penetration, inoculation of the seedling with FO162 reduced the number of juveniles that developed to adult females significantly (figure 5.4). Conversely, the number of the other juvenile stages was higher in roots treated with FO162 although not significantly different from the control roots. The results demonstrated high levels of FO162 inhibition of *M. incognita* development in the endorhiza after penetration.

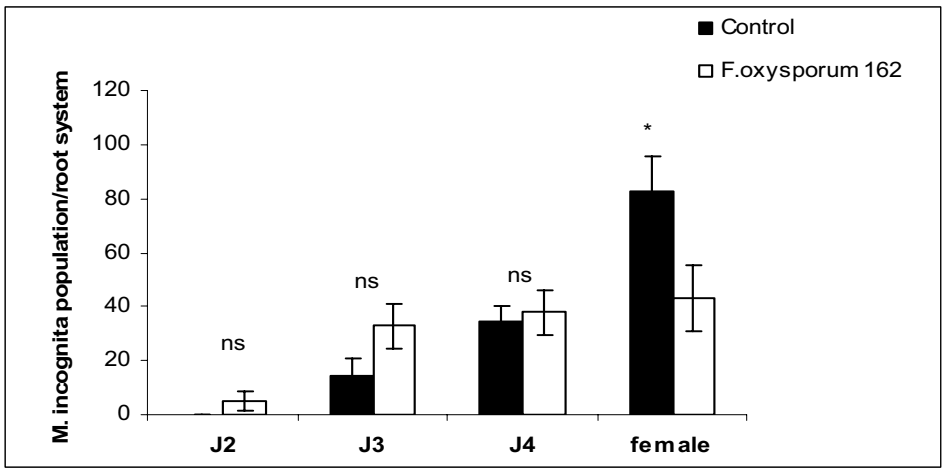


Figure 5.4 Influence of *Fusarium oxysporum* 162 on development of *Meloidogyne incognita* in tomato roots 23 days after synchronized nematode inoculation. Means with (*) are significantly different based on T- test ($P \leq 0.05$; n = 6).

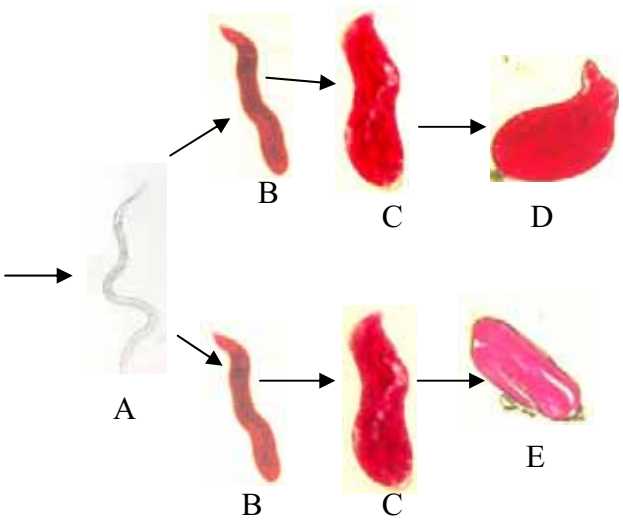


Figure 5.5 Different stages of *Meloidogyne incognita*, A. J2 after hatching, B. J2 after motling inside the roots, C. J3 D. Females and E. Male before molting.

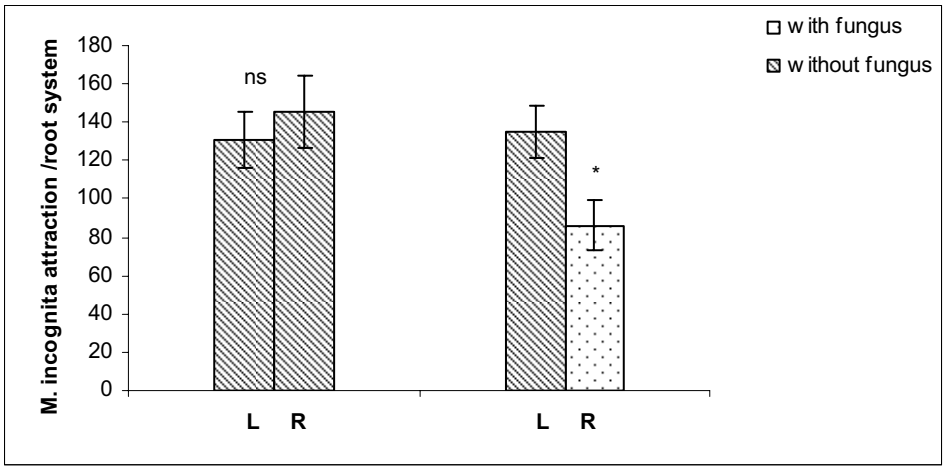
3.2 Juvenile attraction and penetration

The influence of *F. oxysporum* 162 on attraction of *M. incognita* to the root system was conducted in a Linked Twin-pot Chamber experiment. Tomato on the left side of the Linked Twin-pot Chamber was treated with the *F. oxysporum* 162 five weeks before transplanting those on the right remained untreated. Nematodes were inoculated at the middle of the bridge and nematode penetration in both plants was determined.

Nematodes attraction and penetration to tomato was significantly reduced by the presence of FO162 in the root system over a distance of 4 cm two weeks after nematode inoculation (figure 5.6 A&B). No significant difference was detected in attraction to non-treated plants placed in both arms in the same treatment (figure 5.6 A&B).

The number of *M. incognita* that were attracted to the roots of plants treated with *F. oxysporum* 162 was 36-55.9% lower than the control (figure 5.6 A&B). The results demonstrated the presence of root exudate alteration by FO162 that directly affects migratory behaviour in the soil that then directly affects penetration.

A



B

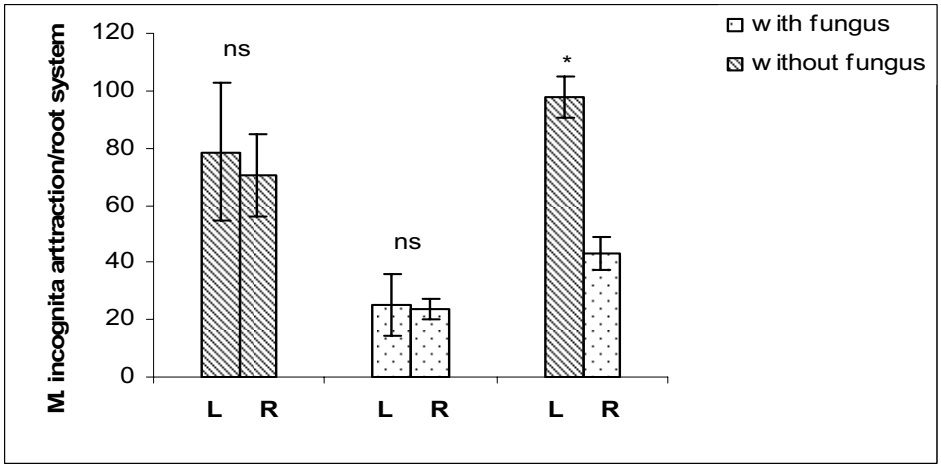


Figure 5.6 Effect of *Fusarium oxysporum*162 on *Meloidogyne incognita* attraction and penetration in the Linked Twin-pot Chamber experiment. L is the pot on the left side and R is the pot on right side of the Twin-pot Chamber. A and B are the first and the second experiments respectively. Means with (*) are significantly different based on T-test ($P \leq 0.05$; n = 8)

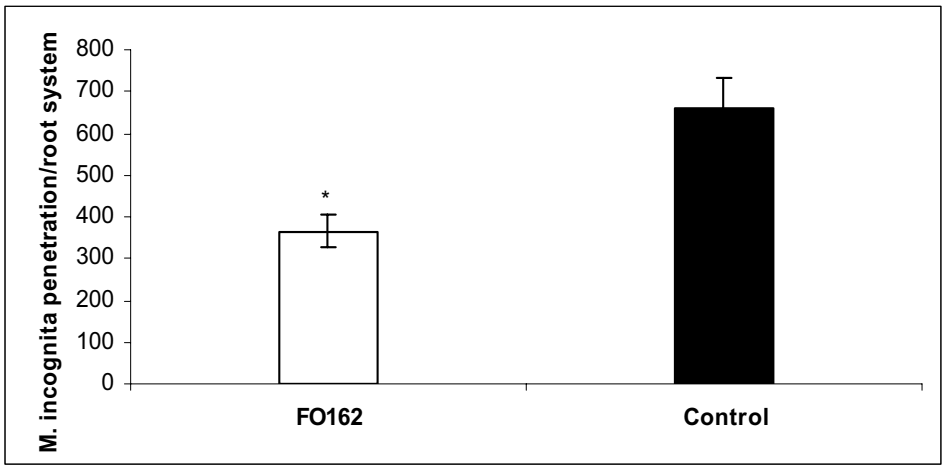
In the non-treated Linked Twin-pot Chamber that served as controls, no difference between attraction to the tomato plants was detected.

As mentioned in the other chapters in this study there also were no significant differences in the fresh weight of roots between the FO162 treated and non-treated tomato seedlings (data not shown).

3.3. Induced systemic resistance

The ability of FO162 isolates to induced systemic resistance was tested in this study by applying the FO162 to the Inducer side of the split root plant and *M. incognita* to the responder side. *M. incognita* infestation in roots in the Responder side of the split-roots after induction by FO162 was reduced significantly when compared to the controls (figure 5.7 A&B). Two weeks after nematode inoculation, *M. incognita* penetration in the responder split-root side was reduced significantly 25.6-44.7% when compared to the roots in the non induced control.

A



B

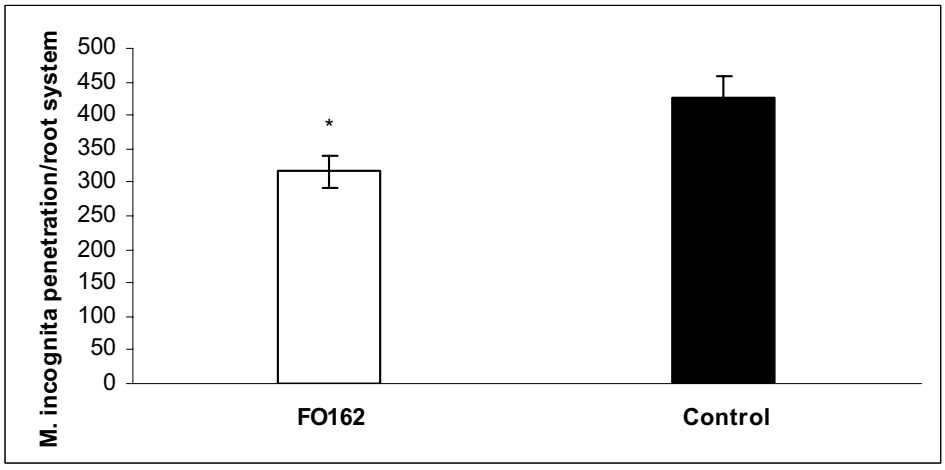
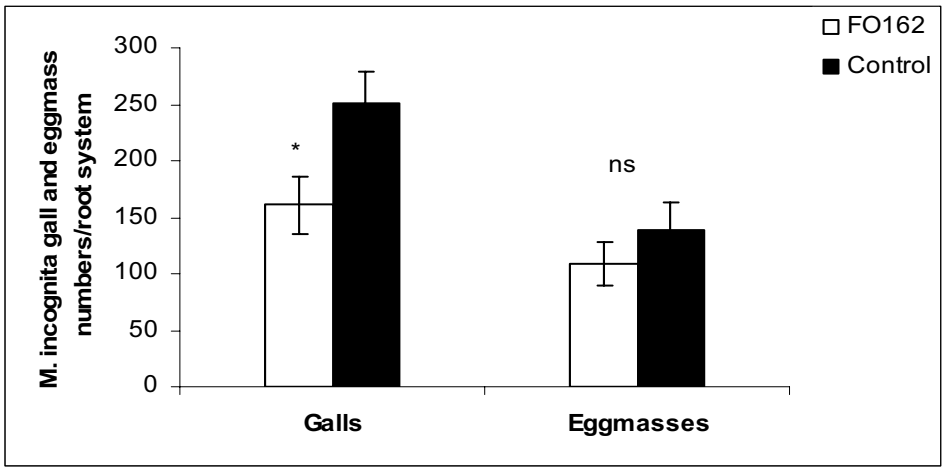


Figure 5.7 *Meloidogyne incognita* penetration in the Responder half of split-root system experiments designed to study the influence of *Fusarium oxysporum* 162 on induced systemic resistance in tomato. A and B are the first and the second experiments respectively. Means with (*) are significantly different based on T- test ($P \leq 0.05$; n = 8)

Long term effects of induced resistance were determined by counting the number of galls and egg masses per each root half 8 weeks after FO162 induction. Number of galls in the Responder split-root side was reduced significantly 26-36 % when compared to the roots in the control. A non-significant reduction of 21-22% in the number of egg masses was obtained in the Responder side following induction with FO162 (figure 5.8 A&B).

However, in this study the reduction in egg mass number was not significantly different ($P \leq 5\%$)) when compared to the control. Moreover, the application of the fungus has no negative effect on shoot or root weight (unshown data).

A



B

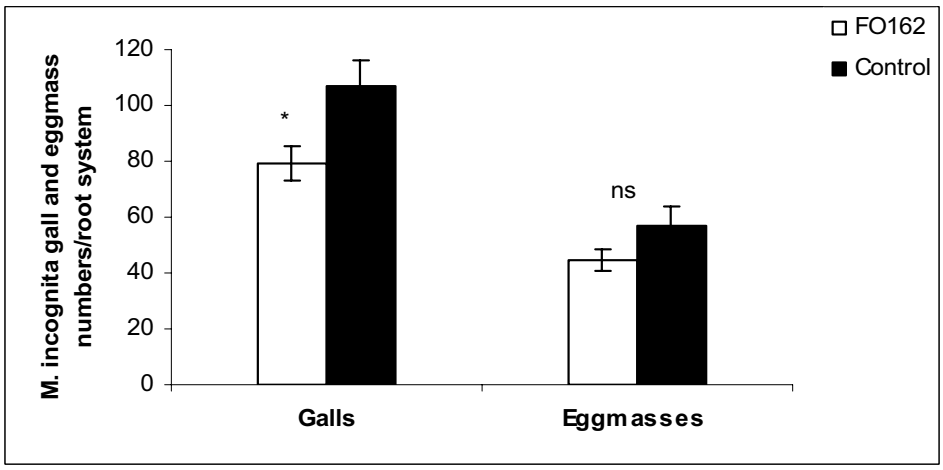


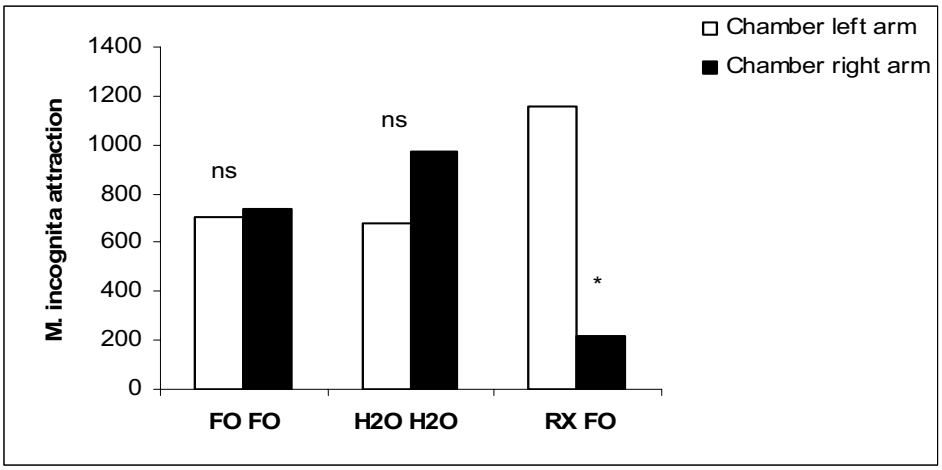
Figure 5.8 Influence of *Fusarium oxysporum* 162 induced systemic resistance on *Meloidogyne incognita* galls and egg mass numbers in tomato roots. A and B are the first and the second experiments respectively. Means with the same letter are not significantly difference based on T- test ($P \leq 0.05$; n = 8).

3.4 Repellent activity of root exudates

One week after nematode inoculation at the centre of the chamber, movement of the nematode away from the FO162 exudate toward the untreated control exudates was significantly increased up to 80% (figures 5.9 A&B). There were no significant differences in migration when both sides of the chamber were inoculated with FO162 root exudate, un-inoculated root

exudates or distilled water (figures 5.9 A&B). The results obtained indicated that exudate of tomato treated with FO162 have a strong repellent activity against *M. incognita* J2.

A



B

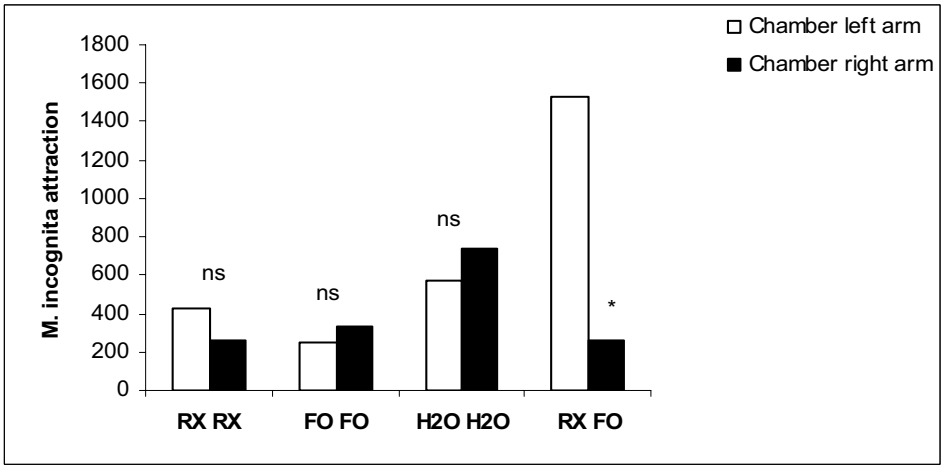


Figure 5.9 Effect of *Fusarium oxysporum* 162 exudates (FO), root exudate (RX), and water (H2O) on *Meloidogyne incognita* movement in soil after 7 days. A and B are the first and the second experiments respectively. Means with (*) are significantly different based on T- test ($P \leq 0.05$; n = 5).

4. Discussion

4.1 Development in tomato roots

In the synchronized nematode development experiment, inoculation of tomato plants with FO162 resulted in inhibition of juvenile development compared to the control. Growth inhibition was attributed to competition for nutrients between the mutualistic endophytic fungus FO162 and the sedentary juveniles.

The number of nematode that remained in juvenile stages in plants treated with FO162, therefore, was higher than in the control plants. This demonstrated that either a direct interaction between FO162 and the nematode or indirect interaction over the plant prolongs the time needed for *M. incognita* to complete its life cycle. The results are important that it should lead to a further reduction in population density over that already attained due to reduced penetration.

Vu (2005) recently studied the influence of different *F. oxysporum* isolated on *R. similis* development stages in banana roots. Here results showed that the *F. oxysporum* isolates did not affect *R. similis* development either when the fungi were inoculated prior to or after nematode inoculation.

4.2 Attraction and penetration

There were major differences in distribution of the nematodes between the two root systems when the plants were treated with *F. oxysporum* 162. Nematode attraction and infestation was reduced significantly by 36–56 % when compared to the non-treated controls in the other pot. These results demonstrate that *M. incognita* migrated away from the treated roots and penetrated tomato that was free from FO162. This means mutualistic endophytic *Fusarium* affected *M. incognita* penetration by reducing *M. incognita* host finding ability. The same results were detected by Vu (2005) using different *Fusarium* isolates against burrowing nematode *R. similis* on banana.

In the present study, non-pathogenic *F. oxysporum* 162 protected tomato seedlings from *M. incognita* infection over distances of 4 cm, indicating that protection did not result alone from antagonism or competition between the fungus and the pest. The results indicate that *F. oxysporum* 162 either produces toxic substances affecting *M. in*cognita behaviour or alters root exudates that control J2 attraction. However, the interrelationship between *F. oxysporum* and the host plant as it affects nematodes has not been thoroughly analyzed. Hallmann and Sikora (1996) suggested that inhibitory compounds produced by the endophyte and transferred into the roots might be involved in the mode of action by producing toxic compounds.

4.3 Induced resistance

In split-root experiments, FO162 demonstrate induced systemic resistance to *M. in*cognita. This finding is similar with that reported by Fuchs et al. (1997) who used *Fusarium oxysporum* strain 47 in bioassays to control the incidence of *Fusarium* wilt in tomato plants. They confirmed that Fo47 induced resistance in tomato against *Fusarium* wilt.

In the present study, *Fusarium oxysporum* 162 induced systemic resistance in tomato plants that affected *M. incognita* attraction and penetration. The application of non-pathogenic endophytic *F. oxysporum* 162 to one side of the Split-root system resulted in a significant reduction of *M. incognita* infestation in the other side of the Split-root system. This clearly shows that protection by non-pathogenic endophytic *Fusarium* is the result of modes of action coupled with induced systemic resistance to *M. incognita* on tomato plants.

In the current study, inoculating the plant with FO162 is effective in the first 2-3 weeks in reducing the nematode penetration, number of galls and egg masses in both direct and indirect contact between the antagonist and the pest. Fuchs et al. (1997) showed that protection was short-lived and had disappeared 6 weeks after challenge-inoculation with FO18. On the contrary, a lower inoculum level of FO47 (i.e. 10^{6} conidia of Fo 47 per ml of substrate) resulted in efficient protection of tomato against *Fusarium* wilt throughout the experiments in another bioassay. The relationship between the inoculum level and nematode control is discussed in more detail in chapter 4 and 6.

The results of this study showed that the fungus has a systematic effect in controlling *M. incognita* in which inoculation of one side of the root with the endophytic fungus and the other side one week later with the nematode, resulted in a reduction in penetration of 25.5 to 44.7%, and 26-36%, 21-22% in the number of galls and egg masses respectively.

Induced systemic resistance in host plants against nematodes was first found using rhizobacteria in potato and tomato (Hasky-Günther et al., 1998; Munif et al., 2001; Hauschild et al., 2004) and was recently described by Siddiqui and Shaukat (2002). Induced systemic resistance can be local, often associated with a hypersensitive response, or systemic when the entire plant becomes protected against later infections. Systemic resistance induced by local infection with a pathogen that causes necrotic lesions is termed systemic acquired resistance (SAR) (Fuchs et al., 1997; Sticher et al., 1997; Oka et al., 2000).

Induction of systemic resistance by non-pathogenic micro-organisms is well known, ISR has been demonstrated against fungi, bacteria, and viruses in Arabidopsis, bean, carnation, cucumber, radish, tobacco, and tomato under conditions in which the inducing bacteria and the challenging pathogen remained spatially separated (Van Loon, 1997). Induced systemic resistance by non-pathogenic *Fusarium oxysporum* against pathogenic *Fusarium* causing wilt diseases on many vegetables have been well documented (Mandeel and Barker, 1991; Alabouvette et al., 1993; Fuchs et al., 1997).

Induced systemic resistance can also be induced by a range of fungi (Mandeel and Baker, 1991; Fuchs et al., 1997; Larkin and Fravel, 1999) bacteria (Van Loon et al., 1998; Reitz and Sikora, 2001; Mwangi et al., 2002; Siddiqui and Shaukat, 2004), nematodes (Ogallo and McClure, 1995), and can be induced chemically (Kessmann et al., 1994; Sticher et al., 1997) with salicylic acid, benzothiadiazole or 2,6-dichloroisonicotinic acid.

Induced systemic resistance was clearly demonstrated to be the main component of the overall mode of action of non-pathogenic endophytic *F. oxysporum* on tomato plants and the key factor affecting nematode behaviour. Vu (2005) used five *Fusarium* isolates that were able to induced systemic resistance against *R. similis* on banana plants by decreasing the penetration rate of *R. similis* into banana roots in both one-pot experiments and Split-root experiments. Biles and Martyn (1989) concluded that watermelon cultivars have different resistances to *Fusarium* wilt when pre-inoculated (induced) with *Fusarium oxysporum* f. sp. *cucumerinum*

or virulent races of *F. o. niveum* 24 or 72 hours prior to challenge with a virulent race of *F. o. niveum*. All inducer treatments significantly reduced wilt symptoms ($P \leq 0.05$). Induced systemic resistance is considered the main mechanism of action of mutualistic organisms in plants against fungal *Fusarium* wilt pathogens (Kroon et al., 1991; Fuchs et al., 1997; Alabouvette, 1998; Koike et al., 2001).

Systemic resistance against fungal, bacterial, or viral pathogens has been analyzed with structurally unrelated compounds. Resistance to these pathogens is systemically induced by specific infections, treatment with microbial cell components, or by a diverse group of structurally unrelated organic and inorganic compounds (Kúc, 2001). Previous studies have shown that ISR triggered by non-pathogenic endophytic *F. oxysporum* protects tomato plants against *Fusarium* wilt disease (Kroon et al., 1991; Fuchs et al., 1997). The later were the first to demonstrate that a non-pathogenic strain of *F. oxysporum* can induce resistance to *Fusarium* wilt in tomato plants and concluded that the non-pathogenic endophytic *F. oxysporum* strains applied on root halves of the plants could delay disease symptoms caused by the pathogen applied separately to the other root halves or to the stem of the plants.

4.4 Repellent

Elongated chambers were used to investigate whether the mutualistic endophyte FO162 has a repellent activity against root-knot nematode. The results showed that the exudates of FO162 significantly reduced the total number of nematodes attracted when compared with root-exudates of control plants. Similar results were recorded by Vu (2005) who suggested that the root exudates or metabolites produced are repellent to *R. similis* on banana.

Inoculating one arm of the chamber with FO162 exudate and the opposite arm with control plant root exudate resulted in nematode juvenile attraction to the control side. This emphasized that this fungus has a repellent activity against *M. incognita.* This was successfully confirmed when compared with chambers in which both arms were simultaneously inoculated with FO162 exudate, root exudate or water. The results showed no significant differences in nematode movement between the two arms.

5. Conclusion

The mechanisms responsible for reduction in root-knot nematode infestation by the non-pathogenic endophytic *F. oxysporum* 162 were studied through different types of experiments. From the results it can be concluded that:

1. In synchronized tests, *F. oxysporum* 162 reduced nematode development in the root system. Indicating competition for nutrients or production of toxic substances in the endorhiza.

2. Nematode attraction was reduced significantly when the roots were colonized by *F. oxysporum* 162, indicating a significant alteration in root exudate pattern.

3. Induced systemic resistance was clearly demonstrated and is an important factor for transmitting mechanisms affecting the nematode throughout the root system.

4. Mutualistic FO162 has a repellent activity against *M. incognita* in the absence of tomato may be due to a toxic compound or due to changes in the root exudate pattern.

Chapter Six

RELATIONSHIP BETWEEN LEVEL OF COLONIZATION OF THE MUTUALISTIC ENDOPHYTIC FUNGUS '*FUSARIUM OXYSPORUM* STRAIN 162' ON *FUSARIUM* WILT RESISTANT TOMATO VARIETIES AND ITS IMPORTANCE FOR CONTROL OF *MELOIDOGYNE INCOGNITA*

1. Introduction

Fusarium oxysporum is a common inhabitant of soils and has a worldwide distribution. The species can be both plant pathogenic as well as non-pathogenic in the soil. All strains of *Fusarium oxysporum* are successful as saprophytes and are able to grow and survive for long time periods on organic matter in soil as well as in the rhizosphere of many plant species. However, some strains that induce root rot or vascular diseases are responsible for diseases on crops of highly economic importance (Olivain and Alabouvette, 1997, 1999; Olivain et al., 2003).These pathogens show a high level of host specificity and based on the plant species and plant cultivars they are able to infect, they are classified into more than 120 formae speciales and races (Armstrong and Armstrong, 1981; Gordon and Okamoto, 1992; Alabouvette et al., 2001).

The penetration process into the root systems is similar for both pathogenic and the non-pathogenic strains. Preferential sites of infection did not exist, and competition between the two types occurred at the root surface, but not for specific sites of penetration (Eparvier and Alabouvette, 1994; Olivain and Alabouvette, 1999).

The wilt-inducing strains are characterised by the ability to enter the plant through the roots and to spread throughout the entire vascular system, inducing yellowing, wilting and finally the death of the plant. To-date host plant inoculation is needed to distinguish between pathogenic and non-pathogenic strains of *F. oxysporum* (Mai and Abawi, 1987; Alabouvette et al., 2001; Olivain et al., 2003).

However, most of the strains in the soil ecosystem are termed non-pathogenic because they are unable to induce disease on a given plant species. This fact plays an important role in the soil microbial balance especially in soil suppression to *Fusarium* wilt. Nevertheless they are mutualistic because they colonize the root tissues of the plant to some extent and live in that tissue (Mandeel and Barker, 1991; Olivain and Alabouvette, 1997; Alabouvette et al., 2001). The evidence that non-pathogenic endophytic *F. oxysporum* isolates were able to reduce *Fusarium* wilt symptoms in plants was obtained in the 1970s (Smith and Snyder, 1971).

A strain of non-pathogenic *Fusarium oxysporum* has been selected for its capacity to reduce the incidence of *Fusarium* wilt on vegetables mainly on tomato under greenhouse and field conditions (Mandeel and Baker, 1991; Alabouvette et al., 1993; Larkin et al., 1996; Olivain and Alabouvette, 1997; Duijff et al., 1998).

Despite this early work, only a few isolates of *F. oxysporum* have been tested in the laboratory and greenhouse for their efficiency as biological control agents on root-knot nematodes (Hallmann and Sikora, 1994a, b). Many strains of non-pathogenic *F. oxysporum* have now been found that reduce endomigratory plant parasitic nematodes on banana (Niere et al., 1998, 2001; Vu, 2005; zum Felde et al., 2005).

Along with studies on biological control activity of non-pathogenic endophytic *F. oxysporum* isolates towards plant parasitic nematodes, the colonization of endophytic fungi is important for control efficacy. Colonization of the host plant by *F. oxysporum* isolates was shown to be important where direct effects of the biocontrol agent on the target organism have been detected or are suspected to be the mechanism leading to disease reduction (Niere, 2001; Vu, 2005). Colonization also is widely believed to be essential for biocontrol of fungi (Handelsman and Stabb, 1996). The first time that microscopic observations have been used to demonstrate that the non-pathogenic strains of *F. oxysporum* are able to colonize plant roots was reported by Olivain and Alabouvette (1997).

Colonization of the endorhiza with the biocontrol agents is important for self propagation and reproduction of the antagonist in the tissue as well as for producing possible parasitism of the

pest or for spreading of toxic compounds in the root tissue (Speijer, 1993). The relationship between the colonization potential of *F. oxysporum* strains on banana and their biocontrol activity against *Radopholus similis* (Pocasangre, 2000; Vu, 2005), and *M. incognita* on tomato plant has been investigated (Hallmann and Sikora, 1994a, b).

For efficient control of plant parasitic nematodes, the mutualistic endophytic biocontrol agents must be pre-inoculated to the plants or the soil, to allow the endophyte sufficient time to colonize the root tissue. Vu (2005) for example reported that inoculation of endophytic *F. oxysporum* fungi after nematode inoculation had no significance effect on *R. similis* control on banana, whereas pre-inoculation gave excellent control. This demonstrated that the endophytic fungi must colonize the root tissue profusely before it is effective as a biological agent against nematode infection. Hallmann and Sikora (1994a, b; 1996) demonstrated that *F. oxysporum* 162 is a mutualistic endophytic fungus that reduces root-knot nematode on tomato. They also showed that at fungal inoculum densities of 2.3 x 10^6 cfu/g root no phytotoxic symptoms occurred.

In addition, good control of fungal wilt disease did not require only a high inoculum density of the biocontrol agent to colonise the roots system but also a high ratio of the non-pathogenic versus the pathogen (Alabouvette et al., 1993; Lemanceau et al., 1993). High colonization of the root with non-pathogenic strains of *F. oxysporum* was required for suppression of *Fusarium* wilt inducing pathogenic strains on tomato. These observations are concordant with the hypothesis of competition between strains of *F. oxysporum* in or on the root. Indeed, both strains colonized the same areas on the root surface and showed great similarities in the colonization process of the internal root tissue (Alabouvette et al., 2001).

Colonization of the root with *F. oxysporum* is considered to be one of the most important aspects affecting the mode of action against plant pathogens. It should be noted that Alabouvette et al. (2001) reported that the same non-pathogenic strain can express several different modes of action. This was especially true for the well-studied Fo47 for which several teams have reported the involvement of: competition for nutrients in soil, competition in root colonization and production of induce systemic resistance. The level of colonization may

influence the mechanism produced at any time. This was the case for the non-pathogenic isolates C5 and C14 isolated by Mandeel and Baker (1991), who attributed the efficiency of the biological control strains to their ability to: penetrate and colonise host tissue without causing pathogenicity, induction of resistance reactions in living cells capable of supplying such reactions, capacity for repeated penetration of moving infection courts to provide continuous induction of resistance and efficient suppressive activity in environments that are conductive for high inoculum potentials of the pathogen.

The mutualistic endophyte FO162 has been shown to colonize the root of tomato varieties susceptible to *Fusarium* wilt but not varieties having wilt resistance. The latter type of varieties is commonly grown worldwide. For that reason the objectives of the following experiments were to study:

1- The level of colonization of *F. oxysporum* strain 162 on a wide range of tomato varieties with both *F. oxysporum* wilt resistance and susceptibility

2- Control efficacy of *F. oxysporum* 162 against *M. incognita* on the same varieties, and

3- The influence of *F. oxysporum* 162 in the absence of *M. incognita* on plant growth promotion.

2. Materials and Methods

The German tomato cv. 'Hellfrucht Frühstamm' which is highly susceptible to *M. incognita* and *F. oxysporum* which was used in earlier studies on biocontrol was used as the standard control.

Six tomato varieties from Seminis vegetables seed-USA (all *F. oxysporum* resistant varieties) and 4 varieties from BEJO ZADEN BV-Holland (2 *F. oxysporum* highly resistant as well as 2 *F. oxysporum* susceptible varieties) were tested (table 6.1).

Table 6.1 Tomato varieties used in the study and their features.

No.	Variety	Germin-ation%	Origin	Company	Disease Resistance
1	FLORIDA 47 R	94	India	Seminis Vegetable Seeds – USA	ASC, F-1, F-2,V-1 (all R), St, (IR)
2	FLORIDA 91 (XP 10091)	94	Thailand	Seminis Vegetable Seeds – USA	ASC, F-2; F-2, St,V-1, (all R)
3	SUNGUARD (XP 10089)	93	Thailand	Seminis Vegetable Seeds – USA	ASC, F-1, F-2,F-3, V-1, (all R), St, (IR)
4	SOLAR SET R	96	Mexico	Seminis Vegetable Seeds – USA	F-1, F-2, St,V-1, (all R), tolerance to heat
5	CROWN JEWEL (XP 01407783)	90	Thailand	Seminis Vegetable Seeds – USA	
6	SUNPRIDE	90	Thailand	Seminis Vegetable Seeds – USA	ASC, F-1, F-2, St,V-1 (all R)
7	P48024		India	BEJO ZADEN BV-Holland	resistant to *Fusarium oxysporum* Fysio 0 & 1
8	P48025		India	BEJO ZADEN BV-Holland	resistant to *Fusarium oxysporum* Fysio 0 & 1
9	P48026		India	BEJO ZADEN BV-Holland	susceptible to *Fusarium oxysporum* Fysio 1
10	P48027		India	BEJO ZADEN BV-Holland	susceptible to *Fusarium oxysporum* Fysio 1
11	HELLFRUCHT/J W FRÜHSTAMM		Germany	Juliwa Enza GmbH & Co. KG - Germany	Susceptible to *F. oxysporum* & N

Disease abbreviations key: ASC= Alternaria stem canker (*Alternaria alternate* f.sp. *lycopersici*); F-1, F-2, F-3=*Fusarium* wilt (*Fusarium oxysporum* f.sp. *lycopersici* races 1, 2, 3); N= Root knot nematode (*Meloidogyne incognita, Meloidogyne javanica, Meloidogyne arenaria*); St= Gray leaf spot (*Stemphylium solani*); V-1 = *Verticillium dahliae* race 1.

Key to resistance information: R = Resistant; IR= intermediate resistant (Seminis Vegetable Seeds, 2006).

2.1 Colonization of *F. oxysporum* wilt resistant and susceptible tomato varieties.

Tomato seeds of 11 varieties were sown (one seeds/pot) in plastic containers measuring 5x4x3 cm. Each pot was filled with a 50 g sterilized sand:soil mixture (2:1, v/v). Immediately after sowing, the endophytic fungus *F. oxysporum* 162 (FO162) was inoculated at 10^8 cfu/g substrate. Pots treated with only distilled water acted as controls. All above mentioned procedures were done under septic conditions in the laminar flow to reduce contamination.

The pots were watered and then transferred to the greenhouse and placed under plastic hoods (60x100x120 cm) to maintain high levels of humidity. The plants were irrigated regularly with distilled water and fertilised one time per week with 14:10:14 N:P:K fertilizer. The experiment was terminated after 3 weeks. The roots were then washed with water to free them of potting substrate and the fresh root weight was recorded.

Fungal re-isolation was determined by surface-sterilizing the roots by soaking them in a solution of 0.5% NaOCl for three minutes, followed by three rinses in sterilized water. Four sections of root approx. 0.5 cm in length were then cut from similar sized roots and then pressed onto PDA to check sterilization success and then mounted onto a new PDA for FO162 colonization determination.

Surface sterilization was considered successful when no fungal colonies developed on the medium used for the imprint. Successful re-isolation was confirmed when the growth characteristics of the outgrowing fungi corresponded with that of FO162. Each treatment consisted of five replicates distributed in a randomized complete block design. A total of 12 root pieces were examined per plant root system and the mean for the 5 plants were calculated.

2.2 Relationship between *F. oxysporum* 162 colonization and control of *M. incognita*

Tomato seeds of 11 varsities (table 6.1) were sown at one seed/plug into commercial 96 plug seedling trays containing seedling substrate, with each plug measuring 4 x 4.5 x 2.5 cm. One of two trays was then treated with FO162 one week after sowing and the other was left untreated. Each plug of the treated tray received 1 ml of a liquid suspension of FO162 containing 10^6 cfu per g seedling substrate. The fungal inoculum was inoculated into the rhizosphere through 3 holes made with a plastic rod around the stem base. Plugs of the untreated tray were treated with tap water.

Seedlings were kept in the greenhouse where they were watered as needed and fertilized weekly. After 5 weeks they were transplanted into pots (7x7x8 cm) containing 400 g of sterilized sand:soil mixture (2:1, v/v), respectively.

A total of seven replicates were used in this experiment, 4 of them were used for re-isolation of the fungus from the endorhiza of the roots (no nematode inoculation) and the other 3 replicates were used to evaluate the efficiency of the FO162 to reduce nematode penetration.

Immediately after transplanting the pots of three replicates were inoculated with 3 ml tap water containing 1000 second-stage juveniles of *M. incognita.* The inoculum was applied into 3 holes around the plant as described for *F. oxysporum* 162. The pots were maintained in a greenhouse at 22ºC ±5 and 16 hours photoperiod.

The experiment consisted of two treatments: 1) *M. incognita* or 2) *M. incognita* with FO162. The experiment was terminated 2 weeks after nematode inoculation or 6 weeks after fungal inoculation. Roots were then washed free of soil with tap water and the root weights recorded. The roots of three replicates were then stained with Fuchsine acid by heating in the microwave for 1.5-2 minutes. These roots were then washed again with water and cut into 1cm lengths and macerated with an Ultra Turrax® T25 in water. The macerate was diluted to 100 ml with water and mixed well by shaking. Counting chambers were filled with 10 ml of

the macerated roots solution and the nematodes that penetrated the root were counted and the total number was calculated. For fungal re-isolation the same procedures were followed as outlined in 2.1.

3. Results

3.1 Colonization of *F. oxysporum* wilt resistant and susceptible tomato varieties.

FO162 colonized all *F. oxysporum* wilt resistant and susceptible tomato varieties as observed three weeks after fungal inoculation. The level of endophytic colonization was found to range between 40–100% among the different varieties (figure 6.1). All the tomato varieties, except one, showed a very high affinity to colonization compared with variety 11 which acted as a standard control in these experiments.

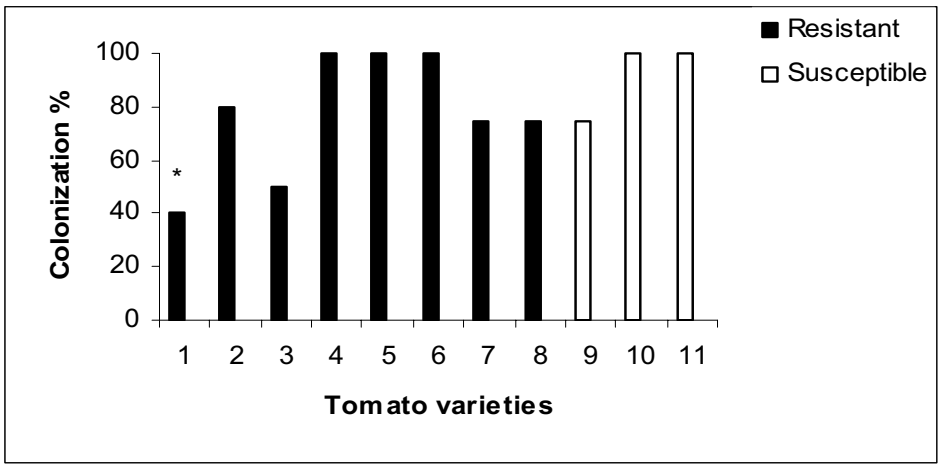


Figure 6.1 *Fusarium oxysporum* 162 colonization of tomato roots in percent, 3 weeks after fungal inoculation. Means with (*) are significantly different based on Tukey test ($P \leq 0.0001$; $n = 5$)

There was no evidence of FO162 colonization in the non-treated tomato roots that served as control nor in the FO162 root print plates (figure 6.2).

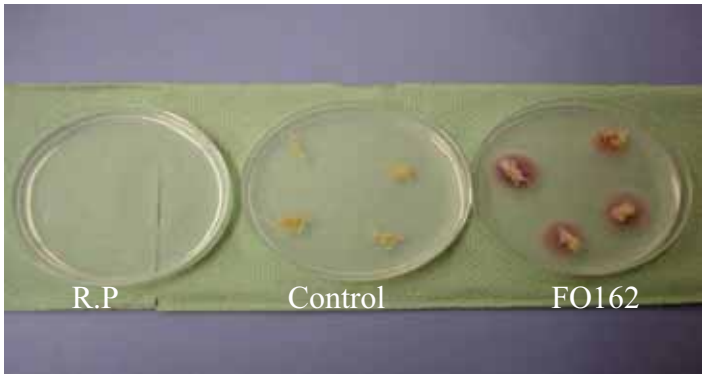


Figure 6.2 Example of re-isolation of *Fusarium oxysporum* 162 from one *Fusarium oxysporum* resistant tomato variety 3 weeks after fungal inoculation compared to the control as well as to the imprint (R.P).

There were an increase in percent root weight in FO162 treated plants that ranged between 26 to 61% compared to the non-treated control roots but the differences were not significant (figure 6.3). However, the average root weight of all the tomato varieties treated with FO162 when pooled was significantly different when compared to the non-treated roots 3 weeks after fungal inoculation (not shown data).

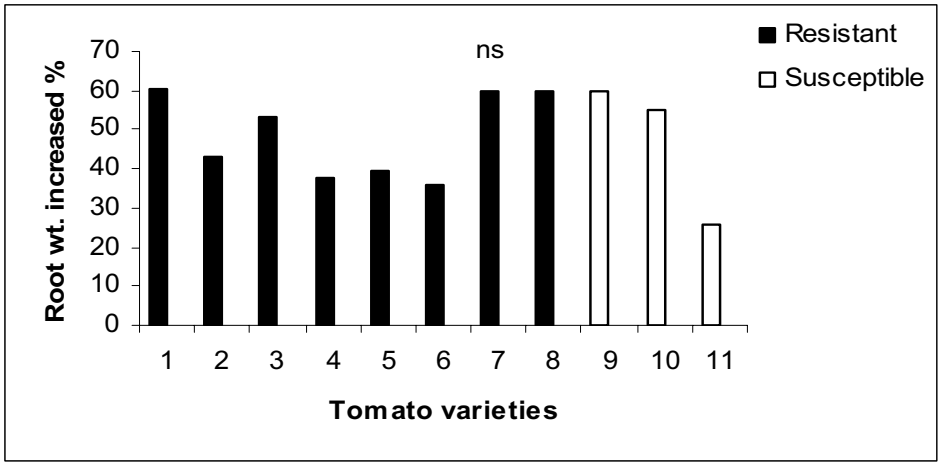


Figure 6.3 Influence of *Fusarium oxysporum* 162 on the root weight of 11 *F. oxysporum* wilt resistant 3 weeks after fungal inoculation in percent of the non-inoculated control. NS: not significantly different based on Tukey test ($P \leq 0.0001$; n = 7)

3.2 Relationship between *F. oxysporum* 162 colonization and control of *M. incognita*

The percent colonization of the roots with FO162 decreased (0-40%) eight weeks after fungal inoculation when compared to colonization in the previous experiment which was determined after 3 weeks (40-100%). The fungus was still present in most *F. oxysporum* wilt resistant varieties (figure 6.4)

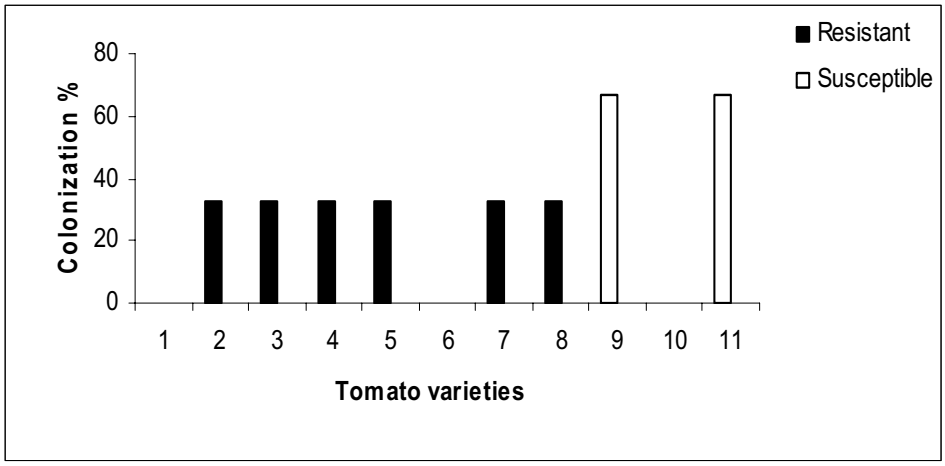


Figure 6.4 *Fusarium oxysporum* 162 colonization percent in tomato roots which are *Fusarium oxysporum* wilt resistant and susceptible eight weeks after fungal inoculation. (n=3)

However, the lack of colonization in some of the varieties after 8 weeks had no effect on the initial reduction in nematode penetration into the roots. The reduction in nematode penetration was significantly different in 5 varieties, 3 of them *F. oxysporum* wilt resistant and 2 susceptible (figure 6.5).

The lack of the detection of FO162 colonization after 8 weeks in some of the varieties may indicate that these varieties are not as sensitive to colonization with age.

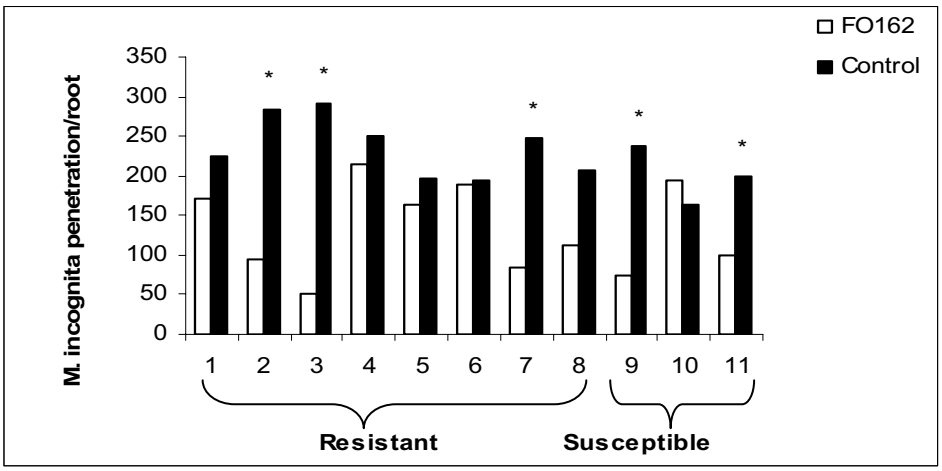


Figure 6.5 Influence of *Fusarium oxysporum* 162 on *Meloidogyne incognita* penetration into tomato roots of 11 varieties. Paired means with (*) are significantly different based on Tukey test ($P \leq 0.0001$; n = 3)

The reduction in nematode penetration was closely related to the percent colonization of the root with the non-pathogenic *F. oxysporum*. Higher percent colonization resulted in lower nematode penetration into the root system. Variety 10, demonstrates this finding clearly in which a lack of colonization (figure 6.4) with the endophytic FO162 resulted in no nematode control. The reduction in nematode penetration into the root tissue reached 82% compared to non-treated control roots (figure 6.6).

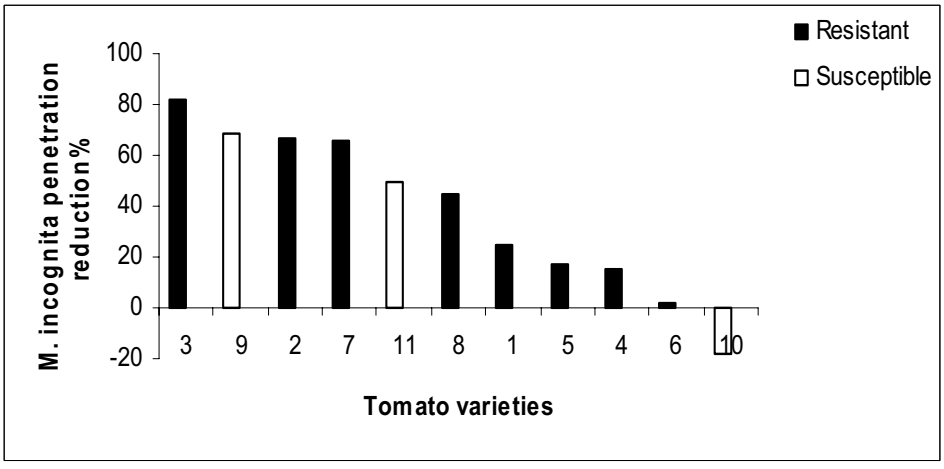


Figure 6.6 Influence of *Fusarium oxysporum* 162 on *Meloidogyne incognita* penetration in percent into 11 tomato varieties in decreasing order of colonization.

4. Discussion

4.1 Root colonization

The objective of this study was to examine the ability of the mutualistic non-pathogenic strain *F. oxysporum* 162 to colonize tomato varieties resistant to *Fusarium* wilt in order to determine their suitability for bio-enhancement toward root-knot nematode.

The endophytic fungus *F. oxysporum* 162 is well known for its ability to colonize the endorhiza of the root tissue of *Fusarium* wilt susceptible varieties of tomato and banana (Hallmann and Sikora, 1994a, 1995; Pocasangre et al., 2000; Niere et al., 2001; Vu, 2005; zum Felde, 2002). The results obtained here are important because it demonstrates for the first time that FO162 has the ability to colonize different tomato varieties even with *Fusarium* wilt resistance.

Colonization of the host plant by the endophytes was shown to be important where direct activity of the biocontrol agent on the disease organism have been detected or are suspected to be the mechanisms of biocontrol (Alabouvette et al., 2001; Niere, 2001).

Studies on colonization of the root by mutualistic *Fusarium* endophytes for the most part are non existent. Most of the literature deals with *F. oxysporum* the wilt pathogen. The only exception is those of *Fusarium oxysporum* strain Fo47 that is used in cross protection studies (Alabouvette et al., 2001).

The first time that microscopic observations were made to demonstrate that non-pathogenic strains of *F. oxysporum* were able to colonize plant roots was recorded by Olivain and Alabouvette (1997). They observed very quickly surface colonization with abundant hyphae at the root surface 24 hours after inoculation of the plant.

In the present study, *F. oxysporum* 162 showed high potential to colonize not only the *Fusarium* wilt susceptible varieties but also wilt resistant varieties. Colonization ranged from

40-100% 3 weeks after fungal inoculation. There were basically no significant differences in the level of colonization between resistant and susceptible varieties except one when compared with the standard susceptible variety that served as a control. There was also no evidence of the presence of FO162 in the non-treated tomato roots of control plants indicating the absence of outside contamination. Niere (2001) and Vu (2005) found that there were no significant differences in the frequency of isolation of *F. oxysporum* from banana plants among the treatments and none of the treatments differed from the control. Vu (2005) demonstrated that 40-45% of the banana root tissue sampled was colonized with *F. oxysporum* isolates 2 weeks after fungal inoculation. There were no significant differences among the different *F. oxysporum* isolates tested. Pocasangre (2000) demonstrated that different endophytic *Fusarium* isolates were found to be present in 45-70% of the tissue sampled on different banana cultivars.

Similar results were reported with non-pathogenic endophytic *Fusarium oxysporum* isolated from eight month old banana plants in the field (Niere, 2001). He detected *F. oxysporum* colonization ranging between 38–49% and 60-100% of the samples from both root and corm tissues of banana one month after fungal inoculation, respectively.

There is apparently no preferential zone for colonization by non-pathogenic strains of *F. oxysporum* on tomato. However, 24 hours after the fungal inoculation, colonization is mainly observed in the zone with root hairs. Hyphae can be observed elsewhere on the root surface and even along the root tip 48 hours after fungal inoculation (Olivain and Alabouvette, 1997; 1999).

In this study, re-isolation of the fungus from the internal root tissue 8 weeks after fungal inoculation decreased up to 50% compared with percent re-isolated after 3 weeks. Colonization could not be detected in some of the varieties after 8 weeks. Similar results were obtained by Niere (2001), who re-isolated *F. oxysporum* 1 or 5 months after fungal inoculation from Gros Michel banana plants. He showed that re-isolation frequency decreased significantly after 5 months.

Eparvier and Alabouvette (1994) demonstrated that competition occurs between pathogenic and non-pathogenic *F. oxysporum* for infection of the root tissues susceptible to colonization. This competition could affect the intensity of root colonization and/or the activity of both pathogen and the non-pathogen. Mandeel and Baker (1991) employed a strain (C5) of *F. oxysporum* that was unable to penetrate and colonize the root of cucumber and consequently was inefficient in biological control against pathogenic strain of *Fusarium* causing wilt. To increase the colonization of the rhizosphere by the biocontrol agents, biocontrol agents inoculated as a combination of isolates to control plant parasitic nematodes and soilborne plant-pathogenic fungi may be needed (Meyer and Roberts, 2002).

4.2 Relationship between colonization and nematode control

In this study, inoculation of the roots with the endophytic fungus FO162 reduced nematode penetration into the roots of all tomato varieties. The reduction in nematode penetration was closely related to percent colonization of the root. The reduction in nematode penetration reached 82% compared to non-treated control roots.

There was still a strong relationship between FO162 colonization of the root endorhiza and nematode infection 8 weeks after the fungal inoculation. The results demonstrated that even when colonization was low control was active.

Working with non pathogenic *Fusarium oxysporum* for cross protection against *Fusarium* wilt Olivain and Alabouvette (1997) assumed that intense colonization of the root surface occurs very quickly after inoculation and constitutes a physical barrier preventing direct contact of the plant pathogen with the root surface. Surface colonization also presents intense competition for root exudates; therefore, the pathogen might not find the nutrients required for propagule germination. However, the colonization of the root surface, although intense, is never complete, and some non-colonized areas might allow the pathogen to reach the root (Olivain and Alabouvette, 1997). The presence of the endophytic fungus and re-isolation of the biocontrol strain at high levels is not necessary for long–term biological control of *R. similis* as suggested by Niere (2001).

Habte et al. (1999) carried out a study to evaluate the effectiveness of three species of arbuscular mycorrhizal (AM) fungi to suppress the plant parasitic nematode *Meloidogyne incognita* in white clover (*Trifolium repens* L.) in a greenhouse. Their results indicated that nematodes caused the greatest damage when plants were not colonized by the fungi. The degree to which mycorrhizal fungi reduced nematode damage varied with the species of mycorrhizal fungus. The effectiveness of the mycorrhizal fungal species in suppressing nematodes was not related to the degree to which they colonized roots or their effectiveness in enhancing host growth. Similar results were obtained by Saleh and Sikora, (1984) who resulted that root colonization of cotton with *Glomus fasciculatum* led to a significant reduction in *M. incognita* egg. However, fungal root colonization associated with this reduction exceeds 90%.

Bao and Lazarovits (2001), showed that mycelia of the pathogenic strain of *F. oxysporum* f. sp. *lycopersici* were rarely observed in sections from roots that had been inoculated with both non-pathogenic *F. oxysporum* strain 70T01 and pathogenic *F. oxysporum* f. sp. *lycopersici*, particularly in the regions where strain 70T01 had colonized.

Excessive colonization of the roots with the non-pathogenic strain prior to invasion by the wilt pathogen was considered of great importance in the direct interaction with the pathogen for the reduction of pathogen infection and thus disease reduction (Bao and Lazarovits, 2001) and also may be important for nematode control.

Recently, the interaction between a non-pathogenic and a pathogenic fungus at the root surface of tomato was studied by Olivain et al. (2006) which showed that the pathogenic fungus was much more difficult to detect when the non-pathogenic fungus was introduced at a higher concentration, but the pathogenic one was never totally excluded.

5. Conclusion

From these experiments the ability of *F. oxysporum* 162 to colonize the endorhiza of tomato varieties with and without *Fusarium* wilt resistant was studied. In addition the relationship between FO162 colonization and biocontrol of root-knot nematode was examined. Based on the results the following can be concluded:

1. *F. oxysporum* 162 has a high capacity to colonize the endorhiza of a wide range of *Fusarium* wilt resistant and non-resistant tomato varieties.

2. Colonization is related to reduction in nematode penetration in wilt resistant and susceptible varieties.

3. In some cases long term colonization is no longer detectable and may indicates a lack of long term protection against multiple root-knot nematode.

4. The root weight of tomato varieties treated with *F. oxysporum* 162 increased significantly 2 weeks after nematode inoculation.

Chapter Seven

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