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Biological, chemical and molecular studies on the systemic induced resistance in tomato against *Meloidogyne incognita* caused by the endophytic *Fusarium oxysporum*, Fo162

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In this study, the role of the mutualistic endophyte *Fusarium oxysporum* (Fo162) in inducing systemic resistance in tomato against the root knot nematode, *Meloidogyne incognita,* was investigated at the biological, physiological and molecular level. It was determined whether Fo162 was able to colonize Fusarium-wilt resistant cultivars and simultaneously induced resistance against root knot nematodes. The results showed that Fo162 successfully colonized the endorhiza of 8 Fusarium-wilt resistant cultivars. A positive correlation was detected between Fo162 colonization levels and root-knot nematode control on both Fusarium-wilt resistant and susceptible tomato cultivars. Remarkably, the levels of Fo162 colonization were higher on the majority of these resistant cultivars when compared to susceptible cultivars, also causing a greater reduction in nematode infection. Then the influence of root exudates obtained from tomato plants, pre-inoculated with Fo162, on root-knot nematode attraction or repellency was determined. The results showed that these root exudates of tomato plants affected the behaviour and migration pattern of *M. incognita* J2. The chemical composition of these root exudates was also biochemically evaluated, using RP-HPLC analysis. Fo162 colonization resulted in increasing the accumulation of several different chemical compounds in root exudates of tomato plants which may be responsible for a repelling effect towards the nematode. The defences in Fusarium-wilt susceptible and resistant tomato cultivars, induced by Fo162, against *M. incognita* were further analyzed with respect to its systemic nature and durability. The results showed that Fo162 was able to induce a systemic resistance of both the Fusarium-wilt resistant and susceptible tomato cultivars tested which could still be detected 7 days after physically separating the endophyte. However, due to the experimental procedure, possible additive effects of wound induced defence responses cannot be ruled out with respect to this prolonged reduction in root-knot nematode infection. The biotic induced resistance, caused by Fo162 was compared with the typical induced systemic resistance (ISR) and systemic acquired resistance (SAR), which can be chemically induced using methyl jasmonate (MJ) and salicylic acid (SA), respectively. The results showed that in a split root experiment these abiotic inducers both increased the levels of systemic resistance and reduced the number of galls of *Meloidogyne incognita* on tomato plants, similar to Fo162. The similarities in reducing root knot nematode colonization by using the biotic and abiotic elicitors offered new perspectives for further research on the mechanism underlying the systemic induced resistance by using molecular tools. Alterations in the expression of genes caused by these elicitors were monitored using a tomato genome array. This demonstrated that the chemical elicitors, SA and MJ, and the biological inducer, Fo162, all alter the expression of a great number of genes. The highest number of genes that were altered in expression level was detected within the plants leaves, especially the plants inoculated with Fo162. By selection the genes, of which the expression had altered in the same direction with all three elicitor treatments, the number of potentially interesting genes could be significantly reduced. Although some candidate genes were identified, further research is necessary to confirm the role of these genes in the systemic

resistance against root knot nematodes. The elicitors also affected the expression of genes, whose products are associated with chlorophyll synthesis and water stress, a finding that corroborated with the physiological and biological observations. This validated the relevance of expression analysis studies by genome arrays as a relevant approach in studying the resistance mechanisms induced by biotic and abiotic elicitors in tomato plants.

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Biologisch, chemisch und molekularbiologische Untersuchung von systemisch induzierter Resistenz an Tomate gegen *Meloidogyne incognita* durch den Endophyt *Fusarium oxysporum*, Fo162
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In der vorliegenden Arbeit wurde der mutualistische Endophyt *Fusarium oxysporum* (Fo162) in Bezug auf induzierte systemische Resistenz an Tomate, gegen den Wurzelgallennematoden *Meloidogyne incognita*, biologisch, physiologisch und molekularbiologisch untersucht. Zudem wurde ermittelt ob Fo162 in der Lage ist *Fusarium*-welke resistente Tomatensorten zu besiedeln und gleichzeitig Resistenz gegen Wurzelgallennematoden systemisch zu induzieren. Die Ergebnisse zeigen das Fo162 die Endorhiza von 8 *Fusarium*-welke resistenten Sorten besiedeln konnte. Es wurde gezeigt, dass die Fo162 Kolonisierung in *Fusarium*-welke resistenten und anfälligen Sorten positiv mit der Kontrolle von *M. incognita* korrelierte. Bemerkenswert war die Tatsache, dass der Grad der Kolonisierung in welke-resistenten Sorten höher war und die Reduktion der Nematoden stärker als in anfälligen Sorten.
Dann wurde der Einfluss von Wurzelexudaten von Fo162 prä-inokulierten Tomaten auf *M. incognita* Anlockung oder Abstoßung untersucht. Die Ergebnisse zeigten, dass die Wurzelexudate das Verhalten und Bewegungsmuster von *M. incognita* J2 beeinflußten. Die chemische Zusammensetzung der Wurzelexudate wurde biochemisch mittels RP-HPLC ermittelt. Es konnte gezeigt werden, dass Fo162 Kolonisierung die Akkumulation verschiedener chemischer Verbindungen, die eine abstoßende Wirkung gegen den Nematoden haben könnten, positiv beeinflußt.
Die Abwehr von *Fusarium*-welke anfälligen und resistenten Sorten, induziert durch Fo162 gegen *M. incognita* wurde im Hinblick auf ihre systemische Eigenschaft und Standhaftigkeit weiterhin untersucht. Die Ergenisse zeigten das die durch Fo162 induzierte systemische Resistenz in anfälligen und resistenten Sorten, selbst sieben Tage nach physischer Trennung von Endophyt und Pflanze messbar war. Jedoch kann ein additiver Effekt durch Verletzungs-induzierte Mechanismen durch die dauerhafte Nematoden Penetration nicht ausgeschlossen werden.
Die biotisch induzierte Resistenz durch Fo162 durch induzierte systemische Resistenz (ISR) und systemisch aquirierter Resistenz (SAR) ausgelöst, wurde durch Chemiekalien wie Methyl-Jasmonate (MJ) und Salicylsäure (SA) hervorgerufen. Die Ergebnisse zeigen, dass diese abiotische Induktion in Split-root-Systemen das selbe Level an SAR und ISR, und die selbe Anzahl an *M. incognita* Gallen an Tomatewurzeln zeigte, vergleichbar mit Fo162 behandelten Pflanzen.
Die Reduktion von Nematoden mit Hilfe von biotischen sowie abiotischen Faktoren eröffnet neue Forschungsmöglichkeiten, die mit Hilfe von molekularbiologischen Techniken hier untersucht wurden. Eine Änderung der Genausprägung durch diese Faktoren wurde mit dem Tomaten Genom Array durchgeführt. Dieser zeigte, dass sowohl SA, MJ als auch Fo162 die Expressionslevels vieler Gene änderte. Die signifikantesten Änderungen wurden in Blättern von Fo162 inokulierte Pflanzen gefunden. So konnten potentielle Gene identifiziert werden die bei allen Behandlungen (MJ, SA und Fo162) reguliert wurden. Obwohl einige Gene identifiziert wurden, bedarf es weiterer Forschung um ihre Bedeutung in

der systemischen Resistenz gegen *M. incognita* zu bestätigen. Außerdem wurden auch Genexpressions Veränderungen von Genen beobachtet die mit der Chlorophyllsynthese und dem Wassertransport assoziiert,sind. Dies wurde mit physiologische-biologischen Veränderungen in Verbindung gebracht. Diese Studie zeigte das Expressionsanalysen mit Hilfe von Genom Arrays ein wichtiger und relevanter Ansatz sind, um Resistenzmechanismen induziert durch biotische und abiotische Faktoren an der Tomate zu untersuchen.

1. General introduction

1.1. The importance of tomato

Vegetables are one of the most important food crops in the world, supplying human with both energy and a wide array of nutrients. Total world vegetable production was estimated at 916 million tons in 2008 (FAO 2008, Agricultural Statistics).

Tomato *(Lycopersicon esculentum* Mill.) is the most important vegetable grown for human consumption. This crop has increased in importance in countries with rapidly expanding population, e.g. Africa and Asia, where tomato production has increased since 1990 by 32 and 50 percent, respectively (Sikora and Fernandez, 2005).

The global production of tomatoes doubled three times in the last 4 decades (FAO 2003, Agricultural Statistics) which is an indication of their importance as a major food crop. World production of tomato (Tab. 1) covers approximately 4 million hectares of arable land with production estimated at 100.5 million tons and valued at 5-6 billion US$ (Costa and Heuvelink, 2005).

Country	Production (million tons)	Area harvested (ha)
China	25.9	1.105.153
USA	12.3	177.000
Turkey	9.0	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	3.0	112.000
Russia	2.2	160.000

Table.1.The top 10 tomato producing countries worldwide (Costa and Heuvelink, 2005; FAO, 2003)

Tomato is also a versatile crop that can be classified according to use into fresh market tomatoes and processing tomatoes which are cultivated for industrial canning and processed foods.

1.2. Root-knot nematodes and vegetable crops

Many soil macro and micro organisms are parasites or pathogens of a wide spectrum of vegetable plants where they can cause severe crop losses.

Plant parasitic nematodes are soil borne pests that attack a wide range of economically important crops where they can affect both yield and quality (Noling, 2005). It has been estimated that 10 percent of world crop production is lost as a result of parasitic nematode infection (Whitehead, 1998).

Among the 24 genera of plant parasitic nematodes affecting vegetables, four species of *Meloidogyne*, the root-knot nematode, are the most economically important to production (Sasser and Freckman, 1987). Vegetables in general are known to be extremely susceptible to root-knot nematode attack. Losses in vegetable production due to *Meloidogyne* spp. attack can reach up to 5 percent (Hussey and Janssen, 2002). Therefore, vegetable production in both tropical and subtropical areas is severely limited by nematode infestation (Sikora and Fernandez, 2005).

Of all vegetable crops, tomato in particular, is heavily infected with plant parasitic nematodes and especially with *Meloidogyne* species (Taylor and Sasser, 1978; Sikora and Fernandez, 2005). *Meloidogyne incognita* is the most predominate and widely distributed representative of this genus. This species has 4 races which can infect selectively more than 2000 host plants (Taylor and Sasser, 1978; Trudgill, 1997; Manzanilla-Lopez *et al.,* 2004). The presence of galls on the root system of infected plants is the primary symptom associated with root-knot nematode infection. These galls affect root functions and reduce nutrient and water uptake that weakens the plants, causing wilting as well as nutrient deficiency symptoms.

1.3. Nematode management

Different control methods are used in the integrated management of plant parasitic nematodes on vegetables. Chemical control, solarization, rotation, steam heating and resistant cultivars are management techniques in wide use. However, all of these management tools have limitations.

The use of chemical control which depends on using fumigation or systemic nematicides is often restricted due to high costs and/or adverse effects on environment and human

health. During the last 20 years no new nematicidal compounds have entered the market that are safer and effective at the same time.
The loss of methyl bromide, a broad spectrum biocide that is extensively used in vegetable production, is a major loss for effective nematode management. The use of systemic nematicides is also being limited by their removal from the market due to adverse side effects.

Agricultural practices like crop rotation, flooding, steam and solar heating as well as fallowing that have been traditionally used in integrated nematode management also have become less usable because of pressure on land and economic demands for more intensification and mass-production of crops and the costs involved that reduces profits.

Moreover, the use of resistant cultivars, which should be a major tool in management of root-knot nematodes on vegetable crops, is restricted due to the lack of nematode resistance genes in most cultivated vegetables.

Resistance to root-knot nematode is only used in industrial production of tomato and has limited use for fresh market tomato. The resistance to root-knot nematode is related to a single group of *Mi*–genes (*Mi*1 to *Mi*8) (Yaghoobi *et al.,* 1995; Veremis and Roberts, 1996a,b; Williamson, 1998) . Important is the fact that *Mi*-resistance to root-knot nematode can be broken-down under temperatures above 28 C°. This limiting factor affects many tomato crops grown in warm tropical and sub tropical regions that are affected by virulent nematode races in the field (Roberts and Thomason, 1989). Therefore, new sources of resistance to *Meloidogyne* spp. are still needed to improve nematode management on all vegetable crops (Hussey and Janssen, 2002). Sikora and Fernandez (2005) also been reported also that the Mi-gene based resistance can be broken by new pathotypes.

1.4. Biological management

The limitations on standard control tools for nematode management, mentioned above, in addition to the limited availability of resistant germplasm for root-knot nematode management, makes finding suitable alternative biocontrol agents for integrated management important (Kerry, 1987; Sikora, 1992; 1997; Viaene *et al.,* 2006). Detection and development of biological agents requires a thorough understanding of the mode of action and the nature of biological activity of parasitic nematodes.

Soil, besides harbouring nematodes and plant pathogens, also supports many other beneficial organisms. Many of the antagonists in soil have been screened for their biocontrol activity against different genera of plant parasitic nematodes on a wide spectrum of crops (Stirling, 1991). Some of these beneficial organisms also have been shown to colonize plants without causing disease and are known as mutualistic endophytes (Petrini 1991; Wilson 1995; Stone *et al.,* 2000).

1.5. Endophytes

Mutualistic endophytic organisms can be either bacteria (Chanway 1996; van Wees *et al.,* 1999) or fungi (Hallmann and Sikora 1994a,b., Niere *et al.,* 2001., Olivain and Alabouvette, 1997) and either obligate symbionts or mutualistic. The arbuscular mycorrhizal fungi (AMF) for example are true obligate symbions of the root system of many plants, whereas others are facultative symbionts (Fo162) that can survive on organic matter in soil or inside the roots of host plants (Sikora, 1997).

Schulz and Boyle (2005) classified fungal endophytes into three ecological groups: the mycorrhizal fungi, the balansiaceous or grass endophytes and the non-balansiaceous taxa. The majority of fungal endophytes isolated from over 500 plants belong to a wide array of genera with the most common being: *Acremonium*, *Alternaria*, *Cladosporium*, *Phoma* and *Fusarium* (Schulz *et al.,* 1993, 1995 and 1998)

In 1997 Olivain and Alabouvette microscopically demonstrated the ability of non-pathogenic strains of *Fusarium oxysporum* that control Fusarium wilt, to colonize root tissue of tomato varieties. Endophytic colonization was considered important when direct competition between the endophyte and the fungal pathogen was responsible for biological control (Alabouvette *et al.,* 2001).

Eparvier and Alabouvette (1994) reported that competition between pathogenic and non-pathogenic *F. oxysporum* isolates in roots of tomato plants affected the colonization level and/or the activity of both pathogenic and non-pathogenic Fusarium isolates. The level of colonization of an endophytic fungus is important for control activity towards a pathogen and can be influenced by biotic and abiotic factors.

Furthermore, different strains of the mutualistic endophyte *F. oxysporum* have been reported to be antagonistic against both fungal pathogens and plant parasitic nematodes on tomato (Mandeel and Baker 1991; Alabouvette *et al.,* 1993; Olivain and Alabouvette 1997; Dababat *et al.,* 2008; Dababat and Sikora2007a,b) and more recently against foliar insects (Vidal, 1996 and Roy-Donald, 2010).

Antagonistic activity of other genera and species of mutualistic fungal endophytes has been detected toward the burrowing nematode *Radopholus similis* on banana (Pocasangre *et al.,* 2000; Niere *et al.,*1998, 2001; Zum Felde, *et al.,* 2005; Vu *et al.,* 2006), toward the lesion nematode *Pratylenchus zeae* on maize (Kimenju *et al.,* 1998) and against the root-knot nematode *M. graminicola* on rice (Le, 2006).

In 1994, Hallmann and Sikora isolated fungal endophytes from roots of field grown tomato in Kenya. Many of these endophytic isolates showed biocontrol activity toward the root-knot nematode *M. incognita*. The mutualistic endophyte *F. oxysporum* strain 162 (Fo162) was the most promising isolate and produced high levels of antagonistic activity against *M. incognita* on tomato. They reported a reduction in gall formation between 50 and 75% within colonized tomato plants by the endophyte in greenhouse trials. The same isolate has been repeatedly shown to have strong activity toward *M. incognita* in other investigations (Dababat *et al.,* 2008; Dababat and Sikora2007a,b).

1.6. Interaction between endophytes, pathogens and host plants

In tritrophic interactions between the host plant, fungal pathogen and mutualistic endophyte, different mechanisms of action were reported and considered responsible for protecting the host plants including: competition for nutrients, competition for infection site, cross protection and/ or induction of host plant defences (Alabouvette *et al.,* 1998; Fravel *et al.,* 2003; Fuchs *et al.,* 1997).

Little is known, however, about the exact mechanism of action involved in tritrophic interactions between the plant, the fungal endophyte and the root-knot nematode. There is evidence that metabolites produced by endophytes are involved in some of these interactions and that the substances influence defence signals and regulation of the symbiosis (Schulz and Boyle, 2005). Speijer (1993) and Hallmann and Sikora (1996) demonstrated that fungal endophytes can release toxic compounds that can reduce nematode infection, when good root colonization takes place. Numerous studies have also

demonstrated that colonization of host plant roots by endophytes results in production of growth-promoting factors (Petrini, 1991).

Endophytes that colonize plant roots also enhance the tolerance of these hosts to environmental stresses (Schulz *et al.,* 1999a,b; Tan and Zou, 2001), to fungal pathogens and plant parasitic nematodes through the production of antimicrobial metabolites (Hallmann and Sikora,1996; Schulz *et al.,* 1995, 2002).

Recent investigations have demonstrated that the induction of systemic resistance in plants following the application of mutualistic fungal endophyte is involved in the mode of action responsible for biological control of nematodes (Vu *et al.,* 2006; Dababat and Sikora 2007a), and to Fusarium wilt (Fuchs *et al.,* 1997).

1.7. Scope of the study

The present study was initiated to identify the mode of action involved in the antagonistic interaction between the mutualistic endophyte *Fusarium oxysporum* (Fo162) and the root-knot nematode *M. incognita* on tomato. Biological, chemical and molecular studies on the mechanisms related to systemic induced resistance were conducted.

The objectives of these investigations were to determine the:

1- Biological activity of Fo162 toward *M. incognita* on Fusarium-wilt susceptible and resistant tomato varieties.
2- Influence of root exudates obtained from tomato plants colonized by Fo162 on root-knot nematode juvenile behavior.
3- Identity of chemical compounds in root exudates obtained from Fo162 pre-inoculated tomato plants which might be responsible for altering juvenile movement patterns.
4- Systemic colonization behaviour of Fo162 and durability of the signals related to the systemic resistance pathways
5- Differences between the biotic Fo162 and abiotic elicitors with respect to form of systemic resistance induction toward *M. incognita*.
6- Regulation of gene expression associated with induction of induced systemic resistance (ISR) and systemic acquired resistance (SAR) using the biotic Fo162 and abiotic (SA and MJ) elicitors.

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2. General Materials and Methods

2.1. Endophyte fungal inoculum

Fungal isolate of Fo162 was originally isolated from the cortical tissues of surface sterilized tomato roots, *Lycopersicum esculentum* Mill. cv. Moneymaker in Kenya (Hallmann and Sikora, 1994). The isolate was cultured and reared on potato dextrose agar (PDA) media amended with 150 mg^{-1} streptomycin and 150 mg^{-1} chloramphenicol (DIFCO Company, Germany) and placed in an incubator at 24 °C for 7 to 14 days. Conidia spores from these initial plates were permanently stored in micro-bank tubes (CRYOBANK™, MASTE Group Ltd., Merseyside, UK) at -80 °C to avoid mutation and contamination. Fungal propagation for all further experiments was initiated with spores from this stock. Inoculum was obtained by culturing the fungus on PDA plates. After 2 weeks, the mycelia and spores were scratched from the surface, suspended in water and sieved through 3 layers of cheese cloth. Number of colony forming units (CFU) in the spore suspension was counted using a Haemocytometer slide (Thoma, Germany) and the concentration was adjusted using sterilized tap water. Desired concentrations for experimentation were suspended in 3 ml water and injected 2 cm deep into the rhizosphere using 3 holes made around the stem base with a plastic rod.

2.2. Nematode inoculum

Meloidogyne incognita race 3 originating from Florida was used in all experiments. Nematode inoculum was multiplied on the tomato cultivar Furore grown in the green house at 27± °C. The plants were grown in large heated controlled boxes (150x80x40 cm) filled with a sterilized soil:sand substrate (1:2, v/v). 3-4 weeks old tomato seedlings were transplanted into the infested substrate and fertilized once a week with a 0.3% fertilizer solution (N:P:K, 14:10:14). Nematode inoculum was obtained through extraction of the eggs from 8 weeks old galled tomato roots using 1.5% NaOCl as described by Hussey and Barker (1973). The plants were uprooted and then roots were washed gently with tap water to remove soil particles. The roots were then cut into 1-2 cm pieces and macerated for 20 seconds in a small amount of water using a warring blender (Bender and Hohbein). The macerate was mixed thoroughly for 3 minutes with 1.5% NaOCl solution and shaken by hand to free eggs from the gelatinous matrix. The egg suspension was poured through 4 sieves: 250 µm followed by 100, 45 and finally 25 µm. Eggs were collected on the 25 µm aperture sieve and then washed with tap water to remove excess NaOCl. Egg inoculum

was transferred into a beaker and then agitated for 7 to 10 days in the dark at room temperature (approx. 20C) with a constant supply of oxygen from an aquarium pump to induce juvenile hatching. The active second stage juveniles (J2) were separated from the un-hatched eggs and the non-active juveniles using a modified Baermann dish technique. The J2 in the resulting water suspension were adjusted to 1000 J2/ 3 ml and used as inoculum. The inoculum was injected 2 cm deep into the rhizosphere using 3 holes made around the stem base with a plastic rod.

2.3. High pressure liquid chromatography (PR-HPLC) analysis

Chromatogram analysis of root exudates collected from *F. oxysporum* 162 inoculated and un-inoculated plants was performed on a HEWLETT PACKARD (HP) system using a LiChrospher® C18 reversed phase guard column (250 by 4.0 mm, 5 µm), preceded by a LiChrospher® C18 reversed phase guard column (4.0 by 4.0 mm, 5 µm). The HPLC system consisted of an HP 1050 pump unit, HP 1050 diode array detector, HP 1046A fluorescence array detector, and a 1050 auto sampler which were controlled by ChemStation for LC 3D system. Before samples were injected, the column had been equilibrated with 90% (v/v) water, 0.1% (v/v) trifluoroacetic acid (TFA) (solvent A) and 10% acetonitril (solvent B). After injection, the samples were eluted at a flow rate of 1.0 ml/min using an isocratic flow of 90% solvent A and 10% solvent B for 2 min, a linear gradient to 10% solvent A and 90% solvent B for 28 min, followed by an isocratic flow for 5 minutes with 90% solvent B.

2.4. IGS-RFLP analysis

2.4.1. IGS-PCR fragments ampilification

The PCR Master Mix was prepared in an autoclaved Eppendorf tube kept on ice, by mixing 12 µL 5 X PCR buffer, 5 µL dNTP (2.5 mM), 1.2 µL PNFO primer, 1.2 µL PN22 primer, 0.25 µL Tag polymerase and 38.35 µL autoclaved MilliQ water plus.

To each PCR vial, 60 µL of the PCR Master Mix was added to 2 µL of DNA sample.

All microfuge vials were closed properly with autoclaved plastic caps and then centrifuged for 2 seconds in a micro centrifuge (Labnet International). The microfuge vials were placed on a PCR thermal cycler (T Gradient, Biometric). Amplification was performed by an initial denaturation at 95 °C for 4 minutes, followed by 36 cycles of 95 °C for 1 minute, 52 °C for 40 seconds, and 72 °C for 1.5 minute, and with a final extension cycle of 72 °C for 5 minutes. After the PCR procedure, samples were stored at 4 °C.

2.4.2. Restriction enzyme analysis

In order to characterize the fungal isolates, the IGS PCR amplified fragments, were digested with 3 restriction enzymes: *Alu*I, *Hae*III and *Rsa*I. In a sterile Eppendorf tube, the Restriction Enzyme Master Mix (REMM) was prepared by adding 0.2 µL acetylated bovine serum albumin (BSA, Promega), 2 µL R Buffer (10 X) (Promega), and 0.5 µL restriction enzyme to 0.3 µL autoclaved distilled milliQ water.

For each isolate, 17 µL PCR product were taken and transferred to a new autoclaved microfuge PCR plastic vial and mixed with 3 µL of the prepared REMM. The plastic vials were closed and centrifuged for 2 seconds before incubated at 37 °C for 3 hours. After incubation, samples were stored at 4 °C. For each sample, 5 µL of the digested IGS PCR product were injected into agarose gel electrophoresis to determine the restriction fragment length polymorphism (RFLP) patters.

2.4.3. Gel electrophoresis analysis

The agarose gel that was used in this analysis was prepared with 1 X Tris-Acetate EDTA-Buffer (TAE, AppliChem). 2.5 g agarose (Sigma) was added to 250 ml of TAE buffer and heated for 5 minutes in a microwave (MW800, Continent) at 650 watts. After cooling at approx. 50°C, 2.5 µL of 10mg ml^{-1} Ethidium Bromide (AppliChem) was added. This solution was poured into an electrophoresis tray and left for approx. 30 minutes until the gel had solidified. The gel was subsequently transferred to the gel electrophoresis chamber filled with 1 X TAE buffer. After transferring all samples to the wells of the gel, the electrophoresis analysis was conducted for 60 minutes at 120 Volt. An Ultraviolet transilluminator was used to visualize the DNA bands and pictures were taken using a digital camera (S9500, Finepix, Fuji film).

2.4.4. Phylogenetic analysis

The IGS-RFLP phylogenetic analysis was processed using NTEdit and NTSYSpc Numerical Taxonomy System, Version 2.2 (Exeter software). The presence or absence of markers was scored for each isolate and the results were compiled in a excel file. Prior to calculation of the genetic similarity (GS), the excel files were converted into an NTSYSpc compatible data matrix. For each marker system the pair-wise genetic distances between taxa was calculated using the DICE coefficient of similarity (Dice, 1945). GS matrices were subjected to the unweighted pair group method with arithmetic average (UPGMA) cluster analysis (Sneath and Sokal, 1973) followed by the sequential agglomerative hierarchical nested (SAHN) cluster analysis (Sneath and Sokal, 1973) implemented in NTSYSpc 2.2.

2.5. 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.

Phloxine B (MERCK)

15	mg	Phloxine B
1000	ml	Tap water

2.6. Statistical analysis

Data were analysed according to the standard analysis of variance procedure with 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 3 were analysed using mixed procedure of SAS (2005). Means of levels of the significantly affecting factors were compared using Tukey's studentized range test with SAS.

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3. Influence of Fusarium-wilt host plant resistance on colonization ability and biological activity of the mutualistic endophyte *Fusarium oxysporum* 162 in tomato.

3.1. Introduction

Many isolates of *Fusarium oxysporum* are known to be important plant pathogens that cause sever damage to many host plants (Olivain and Alabouvette 1997, 1999; Olivain *et al.*, 2003). This fungus contains highly specialized strains that have been classified into approximately 120 *formae speciales* and races, based on the plant species and cultivars they infect (Armstrong and Armstrong 1981; Gordon and Okamoto 1992; Alabouvette *et al.,* 2001). The main form of Fusarium disease management is the use of resistant cultivars which provide full season protection in a number of host plants against the different pathogenic formae speciales.

In contrast, the majority of *Fusarium oxysporum* strains in the soil are non-pathogenic and play beneficial roles in the soil eco-system (Mandeel and Barker 1991; Hallmann and Sikora 1994a,b; Olivain and Alabouvette 1997; and Alabouvette *et al.,* 2001). Some of these non-pathogenic strains are considered to be mutualistic endophytes, because they colonize the root tissues of many plants, live in that tissue without causing disease symptoms and simultaneously increase resistance to fungal pathogens, plant parasitic nematodes and insects. Hallmann and Sikora (1994a,b); and Dababat and Sikora (2007a,b) tested the potential of the mutualistic endophyte *F. oxysporum* isolate 162 (Fo162) against the root-knot nematode *Meloidogyne incognita* and obtained significant levels of control. Fuchs *et al.,* (1997) obtained similar results with Fusarium wilt control with non-pathogenic strains on tomato and more recently, Roy-donald (2010) detected systemic biocontrol activity of Fo162 toward whiteflies and aphids on tomato.

It is important to note that all the previous investigations were conducted on Fusarium wilt susceptible tomato cultivars. This factor is significant in that, the majority of tomato cultivars used in the field are resistant to the pathogenic forms of Fusarium. This form of resistance could influence Fo162 efficacy if the mutualistic endophyte is unable to colonize the root systems.

The capability of a mutualistic endophyte agent to colonize the endorhiza of root systems is essential for providing biological control against pathogens, nematodes and insects. Extremely important in control is the level of endophyte colonization, especially when direct competitive effects of the biocontrol agent on the target organism are involved (Handelsman and Stabb, 1996).

The objectives of the following studies were to determine the:

1- Influence of Fusarium-wilt resistant tomato cultivars on the level of colonization of the mutualistic endophyte .*F. oxysporum* 162.
2- Biological activity of Fo162 toward *M. incognita* on Fusarium-wilt susceptible and resistant tomato cultivars.
3- Relation between Fo162 colonization levels and nematode gall formation on tomato plants.

3.2. Materials and methods

3.2.1. Colonization

Six tomato cultivars from Seminis Vegetable Seed company, USA; four from Bejo Zaden b. v., Holland; and one cultivar (which is highly susceptible to *Meloidogyne incognita*) from Juliwa Enza GmbH & Co. KG, Germany, (Table 1) were sown at a rate of one seed per pot (5 x 4 x 3 cm) in 50 g planting mixture containing a sterilized sand: field soil (2:1, v/v) substrate. Immediately after sowing, the endophytic fungus Fo162 was inoculated at a rate of 10^6 cfu g^{-1} substrate (see chapter 2). Control pots were treated with tap water.

Each treatment consisted of 6 replicates in a randomized complete block design. All tomatoes were grown under greenhouse conditions at 22 ± 5ºC with 16 h of supplemental diurnal light per day. The plants were watered as needed, and were fertilized with a slow release formulation (N: P: K, 14:10:14) at 2 g l^{-1} of water. The experiment was terminated 3 weeks after fungal inoculation. The roots were separated from soil by gentle washing with tap water. The roots were then blotted between two paper tissues and fresh weight was recorded. The roots of each replicate were surface sterilized by soaking them in 0.5% solution of NaOCl for 3 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 in each replicate and each piece pressed onto PDA to check sterilization success (root imprint test). They were then mounted onto a new PDA plate to determine the percentage of root segments colonized by Fo162. A total of 20 root pieces were examined per replicate and the mean of 6 replicates was calculated. Surface sterilization was considered successful when no fungal colonies developed on the medium used for the root imprint. Successful re-isolation of Fo162 from root segments was confirmed when the fungal culture characteristics and microscope examination of the fungi growing out of the root pieces corresponded to those of Fo162.

3.2.2. Biological control

To evaluate the influence of the Fusarium-wilt resistance on biological control activity of Fo162, the number of root-knot nematode galls on the susceptible and resistant tomato cultivars was determined. The 11 cultivars were sown into commercial 96 plug seedling trays containing the seedling substrate as mentioned above.

After three weeks, the tomato seedlings were transplanted into pots containing 300g of planting substrate. Tomato seedlings were inoculated with 3 ml of a solution containing $5x10^8$ cfu of Fo162 (see chapter 2), immediately after transplanting. Control pots were treated with 3 ml of tap water. The plants were inoculated with 1000 J2/pot one week after Fo162 inoculation (see chapter 2). The experiment was terminated 3 weeks after nematode inoculation. The roots were then washed with tap water to free them from soil particles and the number of galls was counted. The root systems were then used to evaluate the level of Fo162 colonization using the same procedure as mentioned above.

Table 1: Tomato cultivars, origin, supplier and susceptibility rating.

No.	Cultivar	Origin	Company	Feature
1*	FLORIDA 47 R	India	Seminis Vegetable Seeds, USA	Fusarium resistant
2	FLORIDA 91 (XP 10091)	Thailand	"	"
3	SUNGUARD (XP 10089)	Thailand	"	"
4	SOLAR SET R	Mexico	"	"
5	CROWN JEWEL (XP 01407783)	Thailand	"	"
6	SUNPRIDE	Thailand	"	"
7	P48024	India	Bejo Zaden b.v., Holland	"
8	P48025	India	"	"
9	P48026	India	"	Fusarium susceptible
10	P48027	India	"	"
11	HELLFRUCHT/J W FRÜHSTAMM	Germany	Juliwa Enza GmbH & Co. KG, Germany	"

*These numbers will be used in the results and discussion instead of the cultivar names

3.3. Results

3.3.1. Colonization in absence of root-knot

The Fo162 strain successfully colonized the roots of all tomato cultivars tested regardless of existence of resistance to the Fusarium-wilt pathogen. Colonization percentage ranged from 31 to 75% among the different tomato cultivars. The highest levels of root colonization (approx. 75 %) were detected in the cultivars Sunguard (3), Florida 91 (2) and P48027 (10), respectively (Fig.1).

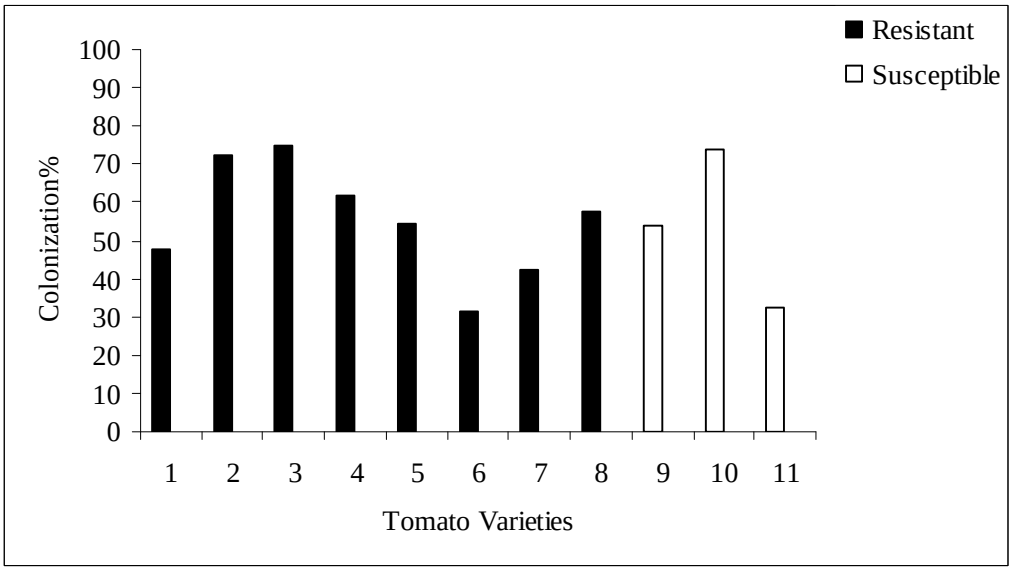

Figure.1. *Fusarium oxysporum* 162 colonization in Fusarium-wilt susceptible and resistant tomato cultivars, three weeks after inoculation (n=6).

3.3.2. Colonization in presence of root-knot

The influence of root-knot nematode infection on colonization levels of Fo162 in Fusarium-wilt resistant and susceptible tomato cultivars was determined in this experiment. Results showed that the highest levels of colonization were detected, as in the previous section, in the cultivars Sunguard (3) and Florida 91 (2). Interestingly, the, levels of Fo162 colonization were higher in the roots of the Fusarium-wilt resistant cultivars when compared to the susceptible cultivars (Fig. 2).

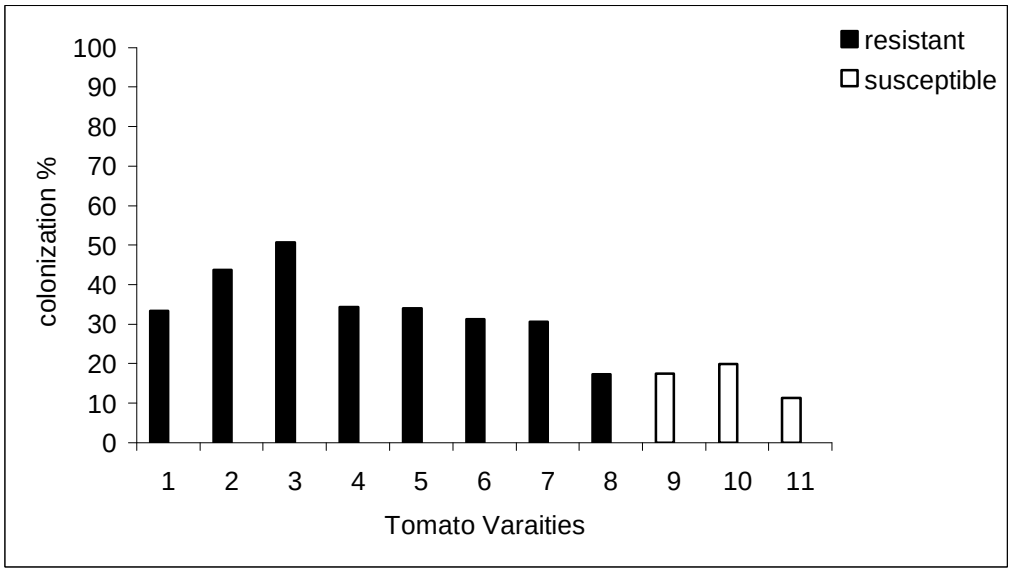

Figure.2. *Fusarium oxysporum* 162 colonization in Fusarium-wilt susceptible and resistant tomato cultivars infested with *Meloidogyne incognita* (n=6).

3.3.3. Biological control

A significant reduction in *M. incognita* gall number per g root material was detected on 4 tomato cultivars, two of which are resistant to Fusarium-wilt (Sunguard (2); and Florida 91 (3)) as well as on two cultivars (P48027 (10) and Hellfrucht (11)) that are susceptible to the Fusarium-wilt, (Fig.3).

Furthermore, the reduction in nematode galls was often related to Fo162 colonization of the root system. High levels of colonization resulted in lower numbers of nematode galls, in the cultivars (2), (3), and (10). Conversely, a high reduction in nematode galls also was detected on the cultivar Hellfrucht (11) in the presence of a lower level of Fo162 colonization.

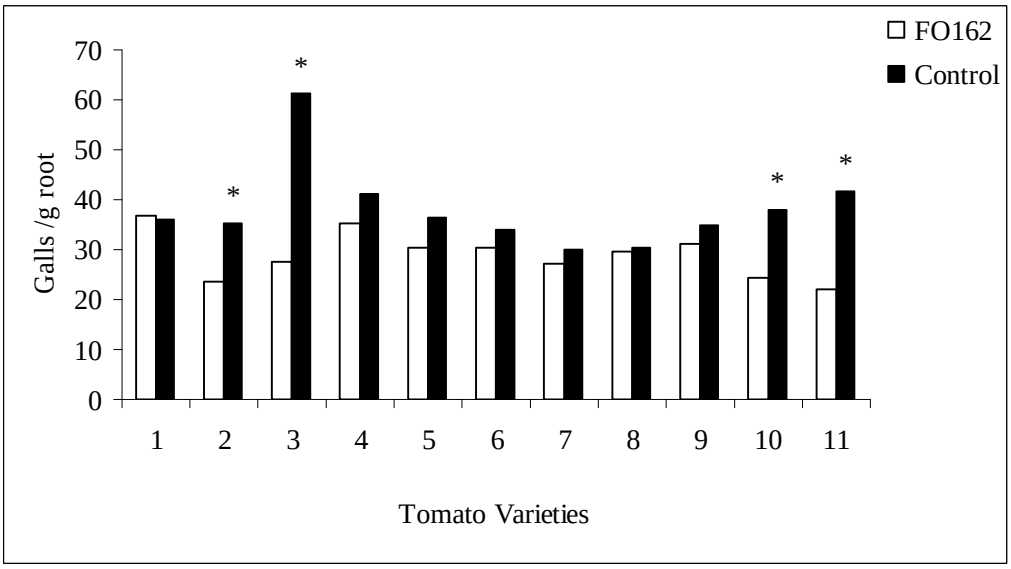

Figure.3. Influence of *Fusarium oxysporum* 162 on the number of nematode galls per gram root within resistant and susceptible tomato cultivars. Paired means with (*) are significantly different based on Tukey test ($P \leq 0.05$; n = 6).

3.4. Discussion

It is well established that the endophytic fungus *F. oxysporum* strain162 (Fo162) has the ability to colonize the root endorhiza of Fusarium-wilt susceptible tomato cultivars as well as roots of other plants (Hallmann and Sikora 1994a; Dababat and Sikora, 2007a,b: Pocasangre *et al.,* 2000 and Roy-Donald, 2010)

Colonization of the endorhiza was found to be important for self propagation and reproduction of mutualistic endophytic antagonists, for parasitism of a pest, for release of toxic compounds as well as for the enhancement of plant defense mechanisms in the root tissue (Speijer, 1993). The level of colonization is often considered to be an important factor influencing both the mode of action involved and the level of control attained (Dababat *et al.,* 2008). Olivain and Alabouvette (1997) were the first to make microscopic investigations which demonstrated that the non-pathogenic *Fusarium oxysporum* isolate (47) was able to colonize the endorhiza of tomato roots. Their study was conducted as in those by Dababat *et al.,* (2008) on Fusarium-wilt susceptible tomato cultivars.

The mutualistic endophyte *F. oxysporum* 162 also has been shown to have biological control activity toward root-knot nematodes on a number of different host plants (Dababat and Sikora 2007c; Vu *et al.,* 2006; Dababat and Sikora 2007a). Alabouvette *et al.,* (2001) also demonstrated that non-pathogenic *F. oxysporum* strain Fo47 successfully colonized tomato roots and affected the incidence of Fusarium-wilt disease through a process that they called cross-protection.

The basis for the present study was to determine whether or not tomato cultivars that have genes responsible for resistance to the Fusarium–wilt pathogen; and that are commonly used in the field for wilt control, have a negative impact on the ability of the mutualistic endophyte Fo162 to colonize the endorhiza of tomato roots and thereby reduce or prevent biological control of root-knot nematode on tomato.

3.4.1. Colonization in absence of root-knot

The results obtained from this study revealed that Fusarium-wilt resistant tomato cultivars do not suppress the ability of Fo162 to colonize tomato root tissue in the absence of the root-knot nematode. Furthermore, the levels of Fo162 colonization were higher on the majority of the Fusarium-wilt resistant cultivars tested when compared to the susceptible cultivars.

3.4.2. Colonization in presence of nematode

When Fo162 was re-isolated from tomato roots that were infested with *M. incognita* one week after endophyte inoculation, the highest levels of Fo162 colonization were detected in the cultivars Sunguard (3) and Florida 91 (2) which are resistant to wilt-Fusarium.

Fo162 colonization levels were lower in tomato plants that were inoculated with root-knot nematode when compared to the non-inoculated plants. The negative impact of the nematode on colonization might be due to the competition for nutrients between Fo162 and nematode in the endorhiza or to abiotic stress present in the greenhouse tests such as temperature.

Eparvier and Alabouvette (1994) demonstrated that competition occurs between the non-pathogenic *F. oxysporum* and the phytopathogens in the root tissue of susceptible plants. This competition affects the intensity of root colonization and/or the activity of both pathogen and the non-pathogen agents.

3.4.3. Biological control

Significant differences in number of galls per g root between Fo162 inoculated and non-inoculated tomato plants were recorded on 4 tomato cultivars. Two of those cultivars are resistant to Fusarium-wilt while the other two are susceptible. Furthermore, the highest level of root-knot nematode control observed on cultivar Sunguard (3) which is Fusarium-wilt resistant. These results revealed that Fo162 was more biologically active on resistant cultivars in reducing gall number and nematode infection.

Moreover, there is evidence that a positive relationship exist between the reduction in nematode galling and the level of endophyte colonization. For example, where the lowest Fo162 colonization levels were detected, also the lowest control potential was observed (cultivar 6, 7, 8 and 9). However, Niere (2001) reported that high level of the endophytic fungal colonization was not required for the maintenance of long-term biological control of *R. similis* on banana.

Investigations conducted with non-pathogenic *F. oxysporum* for cross-protection against *Fusarium*-wilt demonstrated 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 causes intense competition for root exudates. Therefore, the parasite might be prevented from obtaining nutrients required for establishment of infection (Olivain and Alabouvette, 1997; Olivain *et al.,* 2006).

3.5. Conclusions

Based on the finding in the present investigation the following can be concluded:

1- The genes in tomato cultivars that are responsible for providing these cultivars with resistance to the Fusarium-wilt pathogen did not affect the ability of the mutualistic Fo162 to colonize the endorhiza of tomato roots

2- A positive relation was detected between Fo162 colonization levels and root-knot nematode control on both Fusarium-wilt resistant and susceptible tomato cultivars

3- The presence of the root-knot nematode *M. incognita* decreased Fo162 colonization levels in both resistant and susceptible tomato plants.

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4. **Chemical and biological proprieties of root exudates obtained from tomato plants inoculated with *Fusarium oxysporum* strain 162 and their influence on the behaviour of the root-knot nematode *Meloidogyne incognita***

4.1. Introduction

Past research has shown that specific root exudates of host plants play an important role in the infection process and at the same time in host defense responses to different nematodes and pathogens. Ward (1973) demonstrated that nematodes can migrate to specific compounds, such as Cl^- , even in the presence of a high uniform level of another attractive compound such as Na^+. In 1997, Perry reported that plant parasitic nematodes can detect their host plants through chemoreception mechanisms which are dependent on chemotactic signals released by the roots of their hosts. Moreover, Bargmann (2006) demonstrated that *Caenorhabditis elegans* uses chemo sensation mechanisms either to find food or to avoid unfavourable conditions.

The presence of microorganisms in the rhizosphere and in the endorhiza has been shown to influence the attractiveness of host plants to plant parasitic nematodes. Endophytic bacteria have been demonstrated to reduce root-knot nematode penetration on rice and on potato (Padgam and Sikora, 2006; Reitz *et al.,* 2000). Obligate symbiotic arbiscular mycorrhizal fungi also have been shown to alter root-knot nematode host finding behaviour (Reimann *et al.,* 2008). Saprophytic fungi that colonize the endorhiza also have been known to reduce nematode penetration on tomato (Dababat and Sikora, 2007), banana (Zum Felde *et al.,* 2005; Vu *et al.,* 2004), maize (Kimenju *et al.,* 1998) and rice (Le, 2006).

The mutualistic endophyte *Fusarium oxysporum* isolate (Fo162), that has proven antagonistic activity toward root-knot nematodes, was isolated from the cortical tissue of tomato roots (Hallmann and Sikora, 1994). Hallmann and Sikora (1996) and Dababat and Sikora (2007) showed that effective root colonization by Fo162 affected *M. incognita* penetration of the roots. Choice experiments conducted by Dababat and Sikora (2007) demonstrated that tomato root exudates obtained from plants colonised by Fo162 were either less attractive or contained substances that had repellent activity toward *M. incognita* juveniles. The mechanisms involved in alteration of root-knot nematode behaviour in endophyte colonized tomato plants, however, have not been determined.

The objectives of this study were to determine:

1- The influence of root exudates obtained from tomato plants inoculated with Fo162 on root-knot nematode attraction and/or repellency.

2- The chemical components in root exudates responsible for altering root-knot juvenile behaviour.

4.2. Materials and methods

4.2.1. Bioassay – Repellency to unconcentrated root exudates

In the first experiment, seeds of the tomato cultivar (Hellfrucht) which is susceptible to root-knot nematode and to Fusarium-wilt were sown separately in plastic pots containing 250g washed sterilized fine sand. After seed germination (approx. 6 days after sowing) Fo162 spores were inoculated at rate of 10^5 cfu/g sand. The Fo162 spores were produced as outlined earlier (see 2.1.). Plants treated only with deionised water were used as non-inoculated controls. Each of the two treatments was replicated 6 times. Tomato seedlings were watered daily using deionised water: Three days before extracting the root exudates, watering was stopped and the soil in the pots allowed to dry down slowly to concentrate the root exudates.

Root exudates were extracted from Fo162 inoculated and non-inoculated pots 1, 2 and 3 weeks after fungal inoculation. The root exudates were extracted from the sand in the pots after removal of the tomato seedlings by gentle mechanical agitation. The sand from each replicate was then mixed with 100 ml of deionised water in a plastic jar. This suspension was shaken by hand for 2 minutes and then filtered through filter paper (Folded filters 5951/2, Schleicher and Schuell, Germany). The collected filtrate was used for repellent behaviour investigations.

The repellent action of these root exudates on *M. incognita* juveniles (J2) was determined in Petri dishes (standard 9 cm in diameter) containing 1% Agarose gel (DIFCO). A 250 µL drop of the root exudate was applied to one side of the dish and then spread over that half of the plate. The middle zone (1 cm in diameter) was inoculated with ~100 J2. The other half of the Petri dish was left untreated (Fig.1). Petri dishes were incubated at (20°C ± 5 °C) in the laboratory. The number of J2 in each zone was counted under a binocular microscope after 18 hours. This experiment was performed in triplicates.

The root exudates solutions used in this bioassay also were used for HPLC analysis (See 4.2.4.).

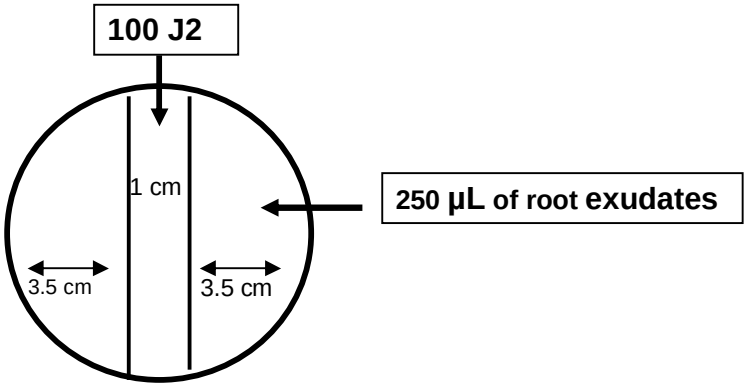

Figure.1. Bioassay test design used to examine *Meloidogyne incognita* (J2) repellency from root exudates obtained from *Fusarium oxysporum* 162 inoculated tomato plants.

4.2.2. Bioassay - Attraction to unconcentrated root exudates

In this experiment, the tomato seeds were again sown separately in plastic pots containing 250g fine washed sterilized sand. Four weeks after sowing, the plants were inoculated with 10^5 cfu/g of Fo162 spores (See chapter 2) and control plants treated with deionised water. Root exudates were extracted as in the previous experiment with a slight modification. The soil of all 6 Fo162 inoculated or control pots were combined and mixed thoroughly by hand with 150 ml deionised water for 2 minutes. The suspension was then filtered through filter paper (Folded filters 5951/2, Schleicher and Schuell, Germany).

A 250 µL aliquot of the root exudates obtained from the control pots was applied to an approximately 3cm wide section on one side of a standard 9cm diameter Petri dish. The root exudate obtained from the Fo162 inoculated pots was applied to the other side (Fig. 2). The same root exudate solutions were used for HPLC analysis (See 4.2.4.).

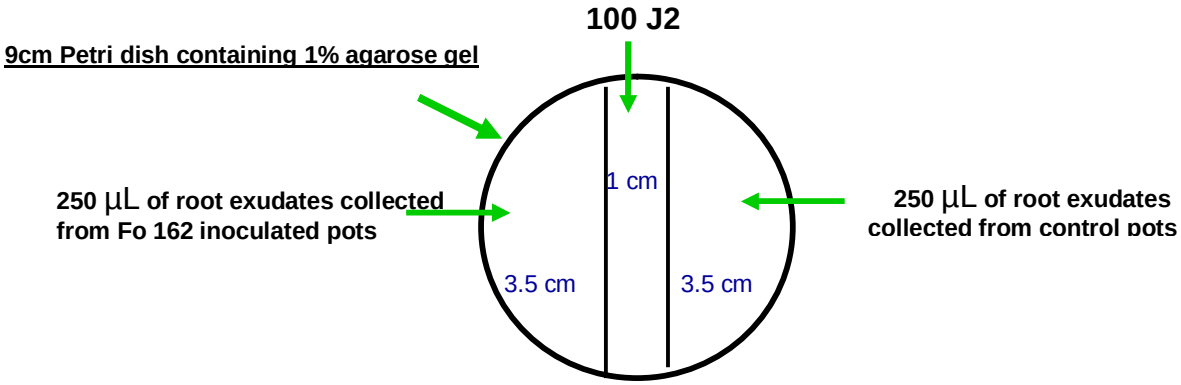

Figure.2. Choice bioassay design used to examine attraction of *Meloidogyne incognita* (J2) to root exudates obtained from *Fusarium oxysporum* 162 inoculated and non-inoculated tomato plants.

4.2.3. Bioassay- Attraction to concentrated root exudates

In this test an attempt was made to obtain a more concentrated root exudate solution, by taking 25 ml of the unconcentrated root exudates solution of each treatment and mixing it with 25ml of ethyl acetate for 2 minutes. After separation, the top phase was transferred to a clean plastic test tube and evaporated to completion under vacuum. The extracted compounds in the tubes were re-dissolved in 250 µL tap water and tested as described in the previous bioassay (4.2.2.).

4.2.4. HPLC analysis

HPLC analysis to study the chemical changes induced by the presence of Fo162 within tomato roots was performed with the same root exudates used in the bioassay-repellency (4.2.1.) and with root exudates used in bioassays (4.2.2. and 4.2.3.). A 20 ml aliquot of the root exudate collected from each pot was mixed thoroughly with 20 ml of ethyl acetate plus 20 µL of trifluoroacetic acid (TFA) in plastic test tubes for 5 minutes. After separation, the top phase was transferred to new tubes and evaporated under vacuum to completion. The extracted compounds were then dissolved in 200uL methanol and 50 µL of each chemical extraction analysed by HPLC (See Chapter 2 for details).

Spectral analysis was conducted to compare the detected peaks with similar retention times in root exudates of the Fo162 inoculated and non-inoculated tomato plants.

4.3. Results

4.3.1 **Bioassay – Repellency to unconcentrated root exudates**

To determine the repellent effect of root exudates obtained from Fo162 colonized plants on *M. incognita* behaviour, root exudates were collected individually 1, 2 and 3 weeks after inoculation as well as from the uninoculated control plants and the J2 placed near the exudates on Petri dishes.

The results showed that significant differences were detected in the behaviour of *M. incognita* J2 moving toward root exudates of Fo162 inoculated plants compared to the control plants when measured 18 hours after exposure on Petri dishes (Fig.3). There also were significant differences in behaviour between the root exudates collected at all three extraction periods (1, 2 or 3 weeks) after fungal inoculation.

Total repellent action was however low ranging between 7 and 10 % percent over the uninoculated control plants 1, 2 or 3 weeks after inoculation with the endophyte.

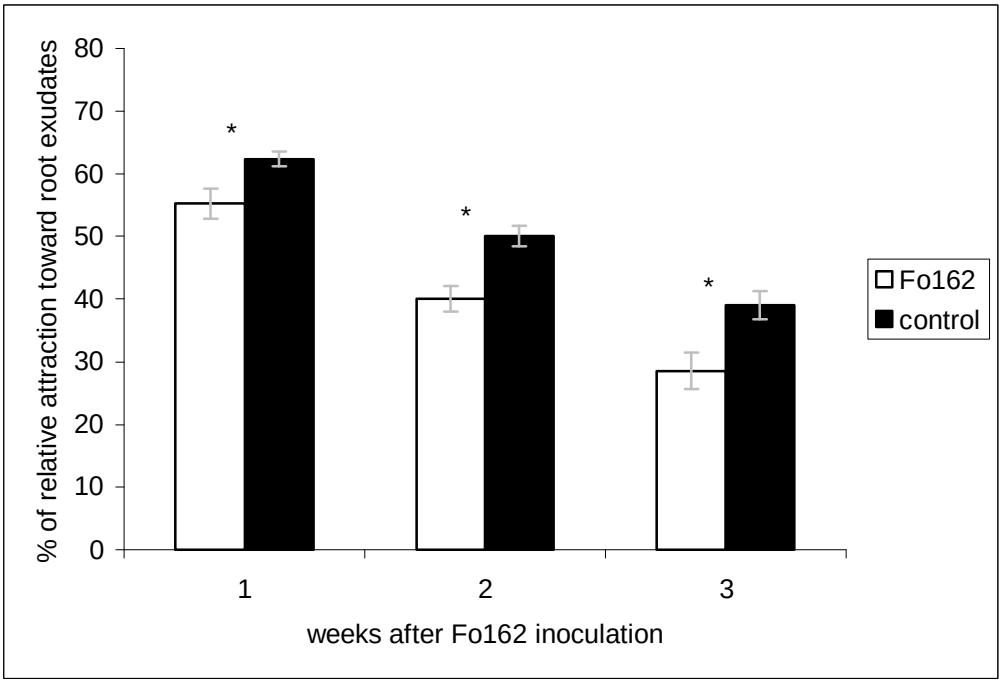

Figure.3. Behaviour of *Meloidogyne incognita* juveniles after 18 hours toward root exudates extracted from *Fusarium oxysporum* 162 (Fo162) inoculated and uninoculated tomato plants 1, 2 or 3 weeks after Fo162 inoculation. Paired means with (*) are significantly different based on Tukey test ($p \leq 0, 05$; n=6).

4.3.2. Bioassay - Attraction to unconcentrated exudates

The biological activity of root exudates obtained from Fo162 inoculated tomato plants on J2 attraction was studied in the second set of experiments using the choice bioassay design described in (4.2.2). The root exudates extracted 1 or 2 weeks after Fo162 inoculation did not have a significant influence on attraction of the *M. incognita* J2 18 hours after test initiation. Conversely, Fo162 root exudates collected 3 weeks after endophyte inoculation, caused a significant decrease in the number of J2 attracted as compared to the uninoculated control exudates (Fig. 4).

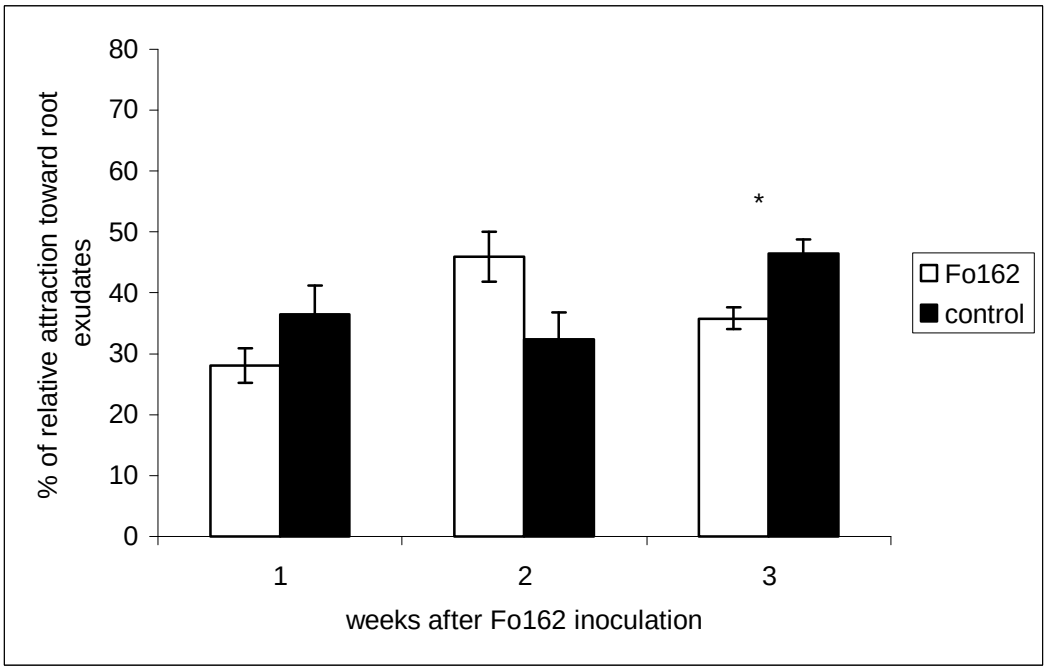

Figure.4. Attraction of *Meloidogyne incognita* juveniles after 18 hours toward root exudates extracted from *Fusarium oxysporum* 162 (Fo162) inoculated and uninoculated tomato plants 1, 2 or 3 weeks after Fo162 inoculation. Paired means with (*) are significantly different based on Tukey test ($p \leq 0,05$; n=6).

4.3.3. Bioassay - Attraction to concentrated exudates

Because of the low level of activity obtained with the unconcentrated root exudates obtained from Fo162 inoculated tomato plants on the behaviour of *M. incognita* J2, a bioassay was conducted with concentrated root exudates to determine if the compounds responsible for attraction are present in low amounts.

The results showed that the concentrated root exudates of Fo162 were either less attractive or more repellent to the J2 when compared to those of the control plants. The percentage of J2 present in the zones inoculated with root exudates obtained from Fo162 inoculated plants was lower 1 or 2 weeks after endophyte inoculation compared to the control after 18 hours of exposure respectively (Fig.5).

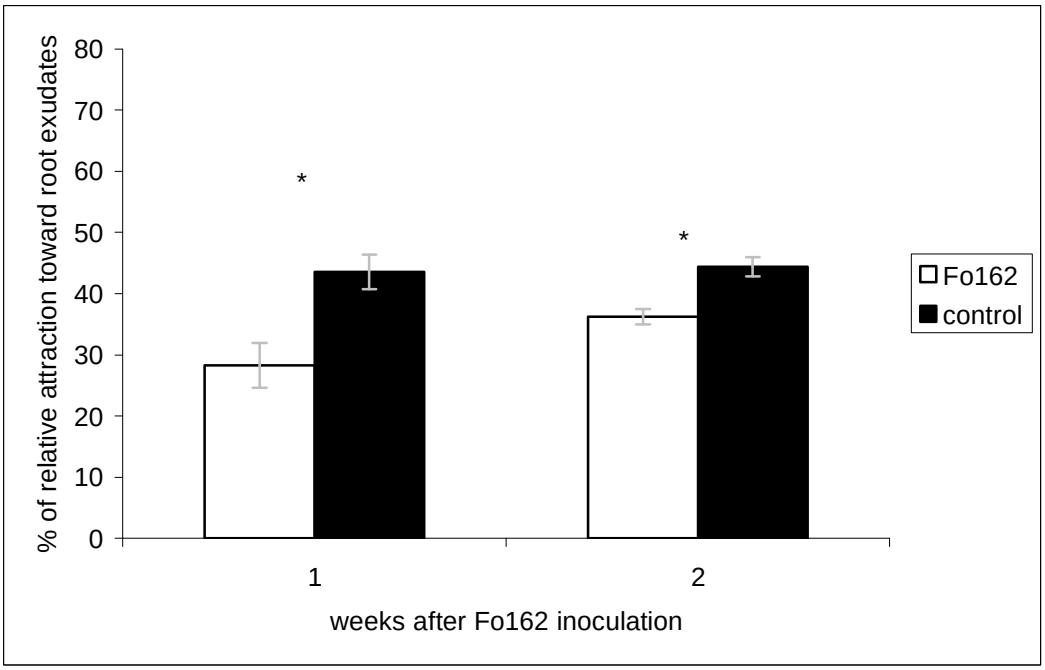

Figure.5. Attraction of *Meloidogyne incognita* juveniles after 18 hours exposure toward concentrated root exudates from *Fusarium oxysporum* 162 (Fo162) inoculated and uninoculated tomato plants 1 or 2 weeks after Fo162 inoculation. Paired means with (*) are significantly different based on Tukey test ($p \leq 0, 05$; n=6).

4.3.4. HPLC analysis

4.3.4.1. Bioassay – Repellency to unconcentrated root exudates

The root exudates used in bioassay-repellency (4.2.1.) were analyzed for activity using the array detector chromatogram at a wave length of 270nm. The differences between root exudates of Fo162 inoculated and control plants were determined. Based on this, the area under the curve of the peaks with retention times of 14, 21, 25, 26 and 29 min were examined (Fig. 6). Similarity of the peaks detected with similar retention times within the root exudates of control and Fo162 inoculated plants was also analysed using spectral analysis (Fig. 7).

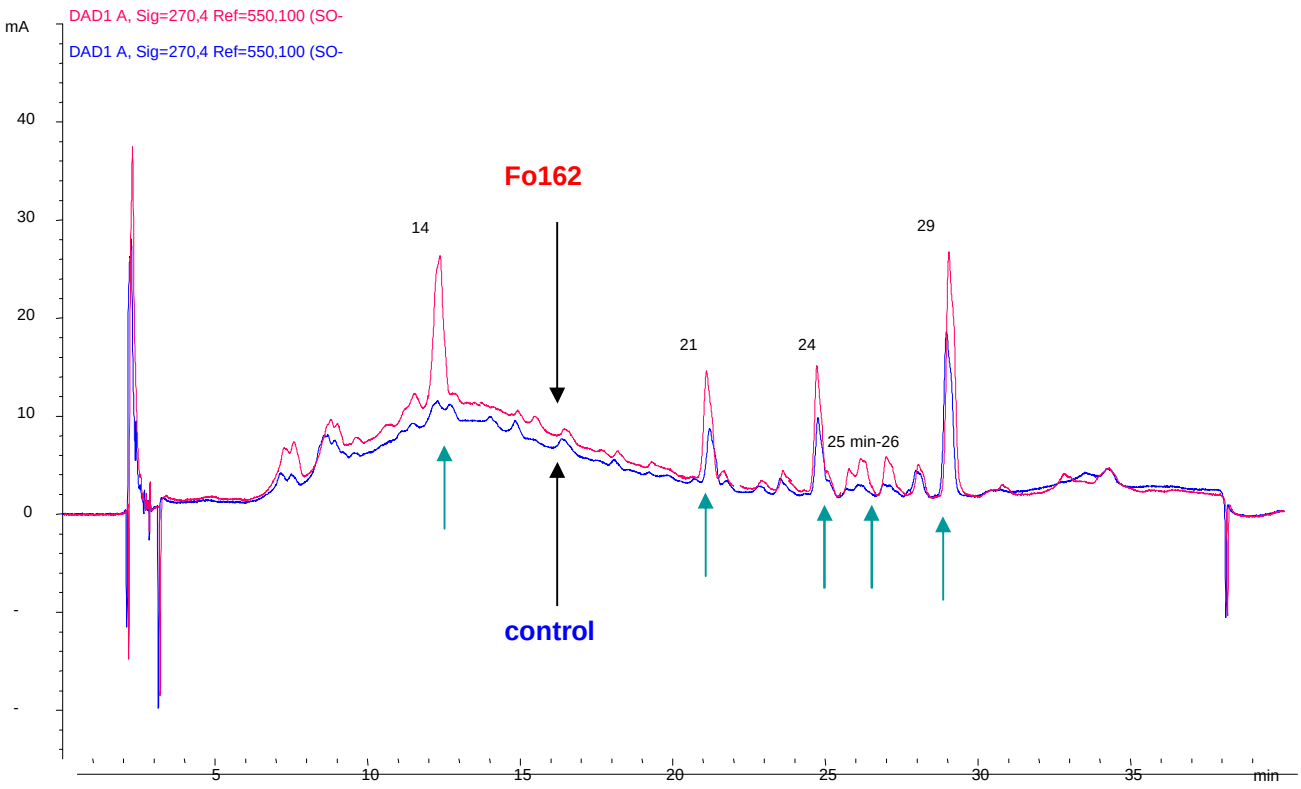

Figure.6. HPLC-chromatogram of root exudates obtained from *Fusarium oxysporum* 162 inoculated and non-inoculated tomato plants recorded at a wave length of 270nm.

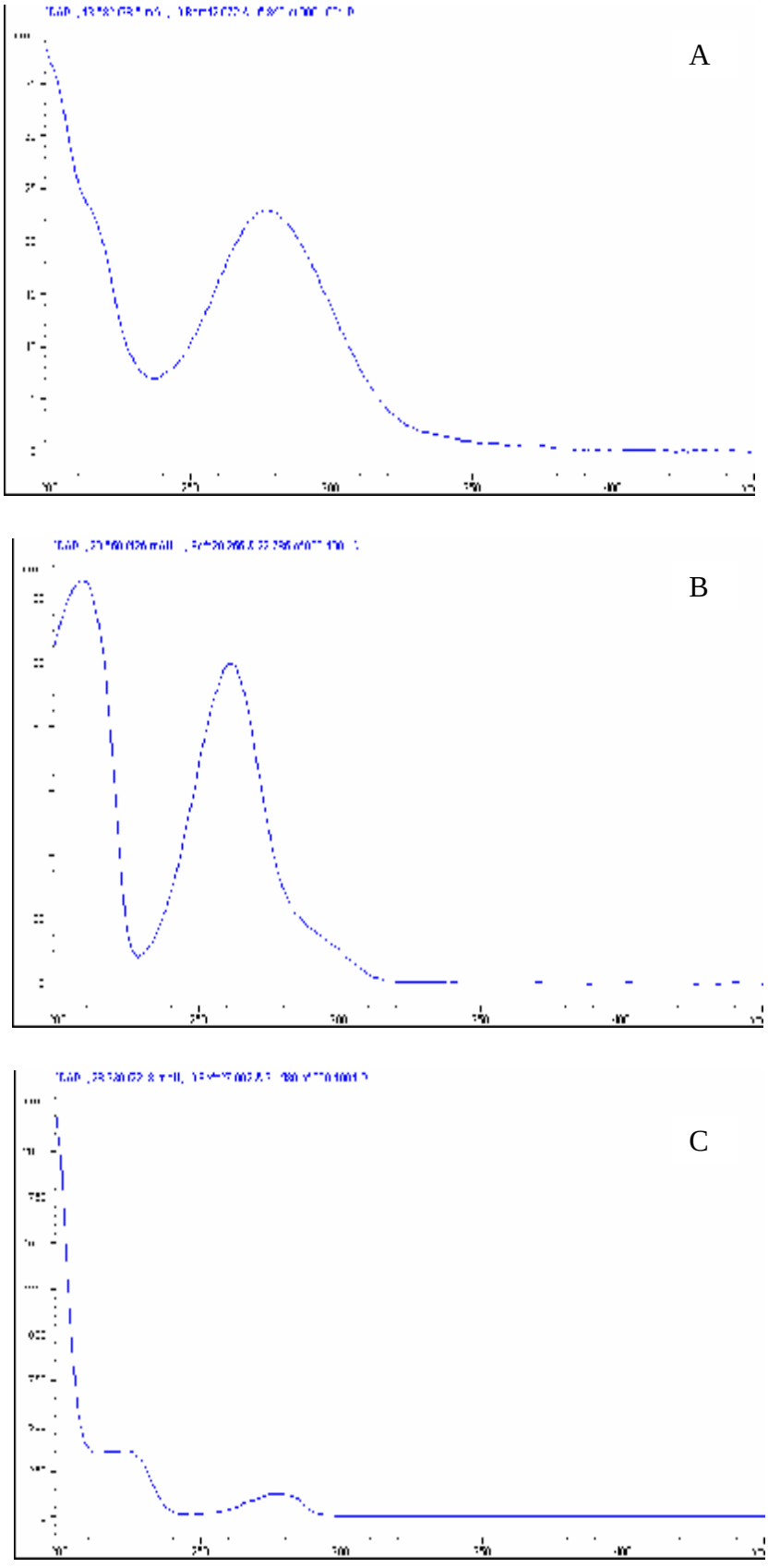

Figure.7. Absorption spectra of the peaks with a retention time of approx. 14 minute (A), 21 minute (B) and 29 minute (C) detected at a wave length of 270 nm.

The results showed that with root exudates collected 1 week after Fo162 inoculation, the accumulation of the compounds detected at the above mentioned retention times was higher in root exudates of Fo162 inoculated plants compared to the control root exudates, (Fig.8). Moreover, the results showed that the compound with a retention time of 14 minutes was present only within root exudates of Fo162 inoculated plants and was not detectable in the root exudates collected from control plants.

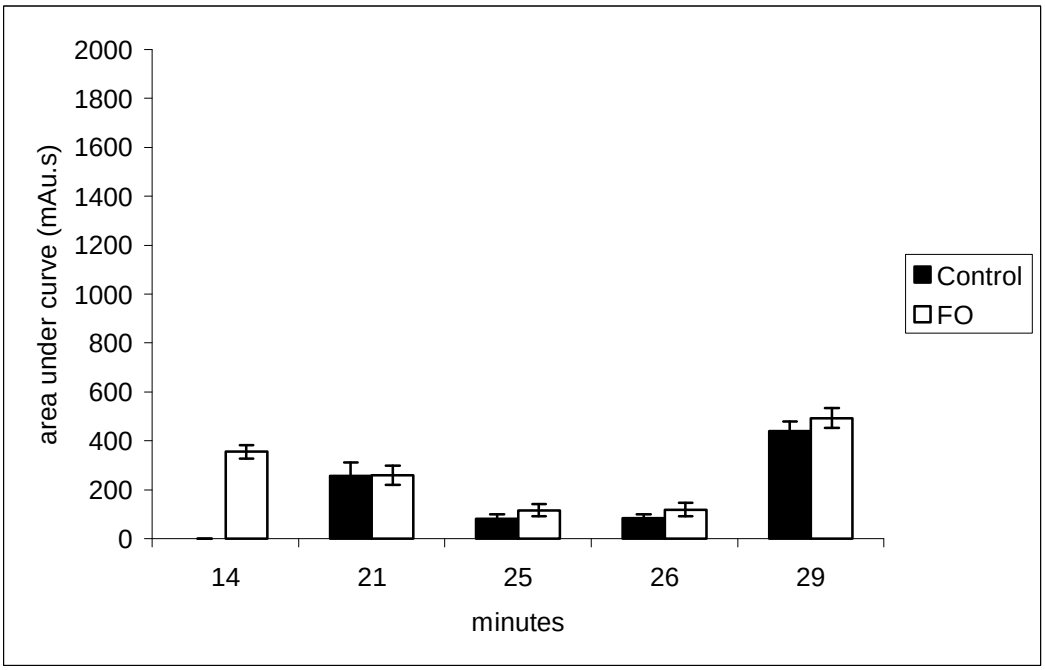

Figure.8. Accumulation of the compounds with the retention time of 14, 21, 25, 26, and 29 minutes detected at 270nm in root exudates obtained from *Fusarium oxysporum* 162 (Fo162) inoculated and non-inoculated tomato plants 1 week after fungal inoculation.

Analysis of root exudates collected 2 weeks after endophyte inoculation showed an increase in accumulation of a chemical compound detected with retention time of 21 minutes. The accumulation of this compound was 3-fold higher in the root exudates obtained from Fo162 inoculated plants compared to the control exudates (Fig.9).

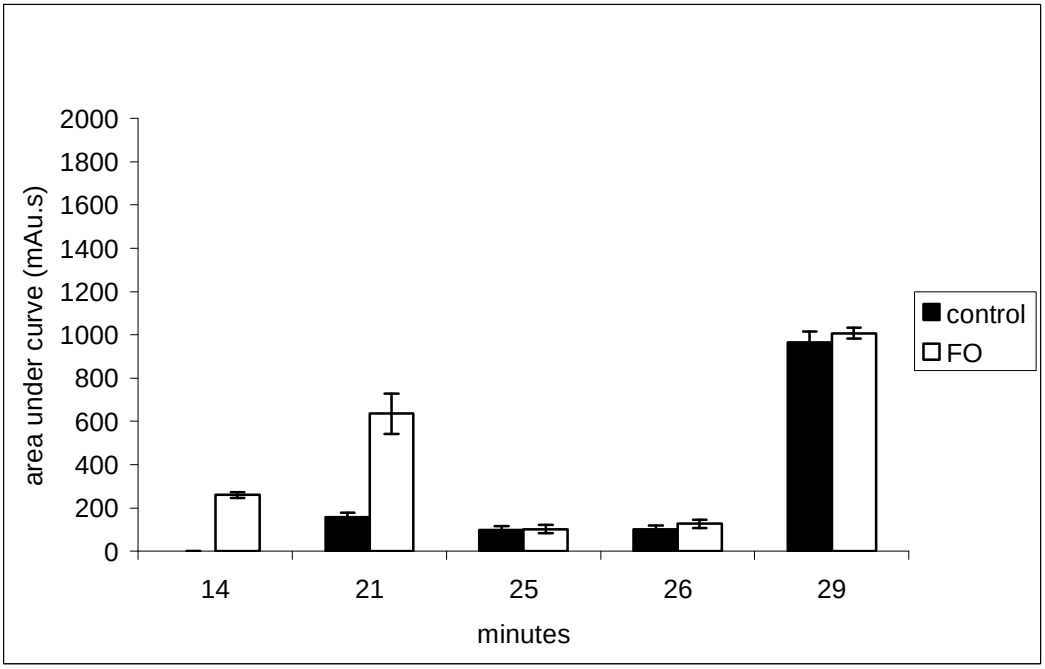

Figure.9. Accumulation of the compounds with the retention time of 14, 21, 25, 26, and 29 minutes detected at 270nm in root exudates obtained from *Fusarium oxysporum* 162 (Fo162) inoculated and non-inoculated tomato plants 2 week after fungal inoculation.

HPLC analysis of root exudates obtained 3 weeks after Fo162 inoculation also revealed significant differences in chemical content between the exudates of Fo162 inoculated plants and the control exudates with respect to the accumulation of the compound detected with retention time of 29 minute, (Fig.10).

The accumulation of the compound detected with retention time of approximately 14 minutes in exudates of Fo162 collected 1 week after endophyte inoculation, decreased significantly in Fo162 root exudates collected 2 and 3 weeks after Fo162 inoculation.

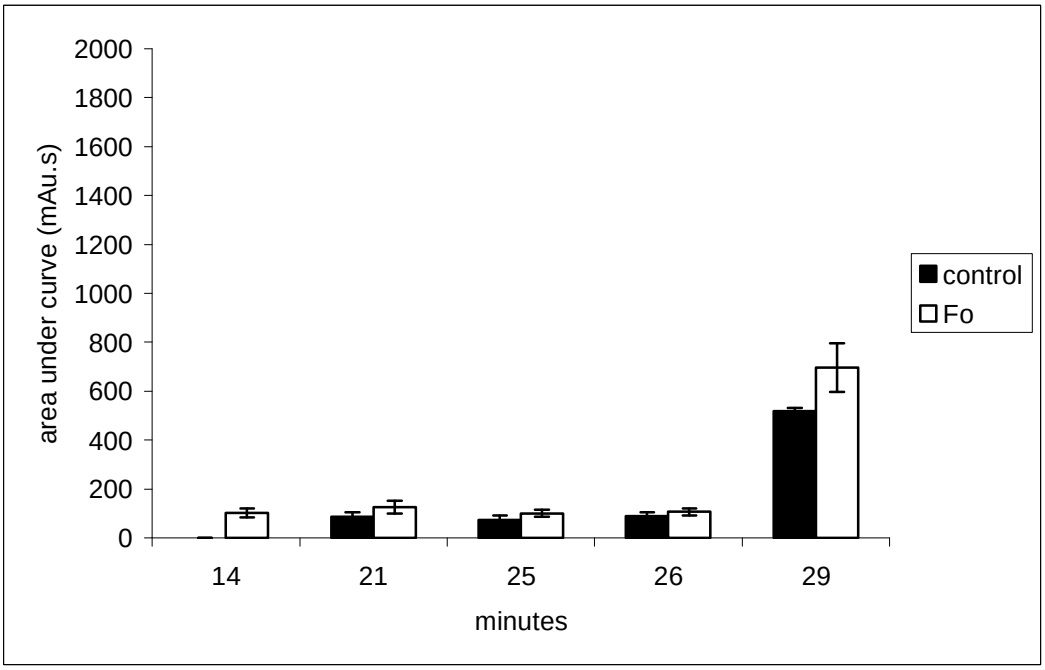

Figure.10. Accumulation of the compounds with the retention time of 14, 21, 25, 26, and 29 minutes detected at 270nm in root exudates obtained from *Fusarium oxysporum* 162 (Fo162) inoculated and non-inoculated tomato plants 3 week after fungal inoculation.

The UV spectrum of the peaks detected in exudates of Fo162 and the control plants at identical retention times were identical (Fig. 7).

4.3.4.2. Bioassay – Attraction to unconcentrated and concentrated exudates

The results obtained from the chromatogram analysis of root exudates collected from Fo162 inoculated and uninoculated tomato plants 1 week after fungal inoculation and used in the behavioural bioassay 4.2.2 and 4.2.3, showed a higher level of accumulation of compounds detected at a wave length of 270nm and retention times of 21, 25 and 26 minutes in Fo162 root exudates compared to the control (Fig.11).

The results also demonstrated that the peak with a retention time of 14 minutes which was detected in the exudates used in the bioassay for repellency was again detectable only in root exudates taken from Fo162 inoculated plants. .

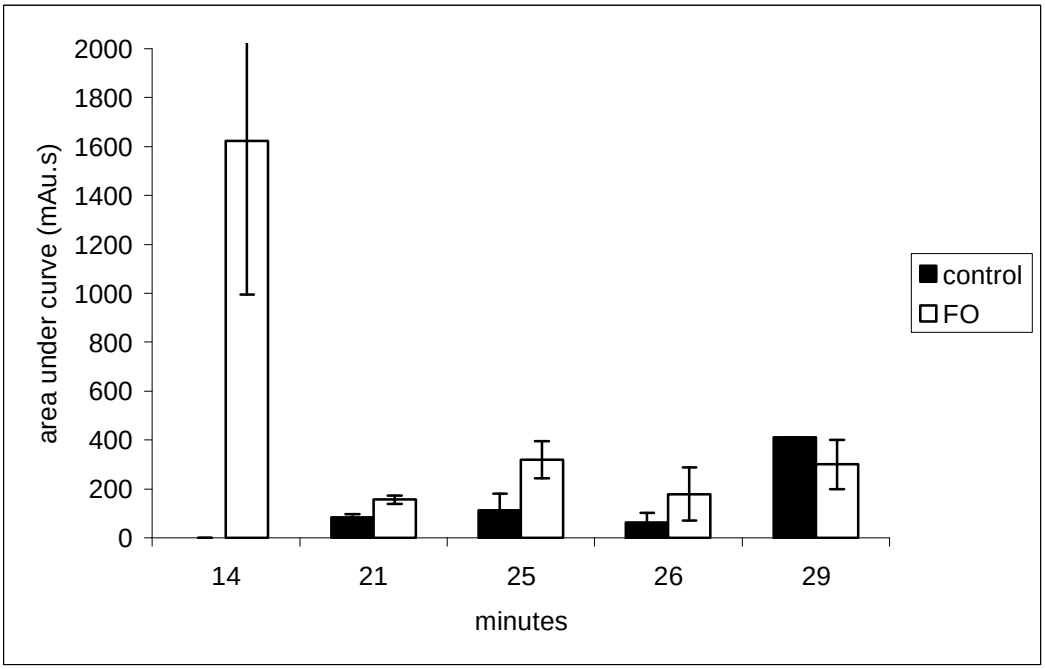

Figure.11. Accumulation of the compounds with the retention time of 14, 21, 25, 26, and 29 minutes detected at 270nm in root exudates obtained from *Fusarium oxysporum* 162 (Fo162) inoculated and non-inoculated tomato plants 1 week after fungal inoculation.

Similar results were observed again in root exudates collected 2 weeks after Fo162 inoculation. The area under the curve of the peaks with retention times of 21, 25, 26 and 29 minutes was larger in the root exudates of Fo162 inoculated plants than in the control exudates (Fig.12). Furthermore, the compound detected with retention time of 14 minutes was only detected in root exudates of Fo162 inoculated plants.

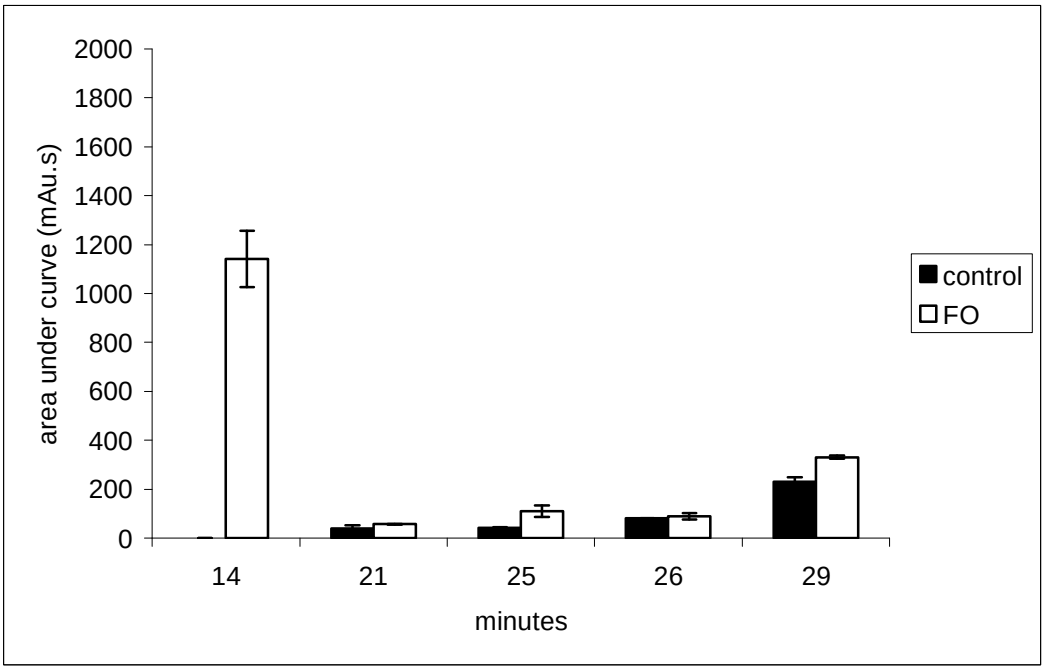

Figure.12. Accumulation of the compounds with the retention time of 14, 21, 25, 26, and 29 minutes detected at 270nm in root exudates obtained from *Fusarium oxysporum* 162 (Fo162) inoculated and non-inoculated tomato plants 2 weeks after fungal inoculation.

Results obtained from root exudates collected 3 weeks after Fo162 inoculation were similar to those obtained from root exudates collected 1 and 2 weeks after fungal inoculation. The differences in the area under curve of the peaks with retention times of 25, 26 and 29 minutes were detected again between the Fo162 exudates and the control exudates as in the previous experiment on repellency (Fig.13).

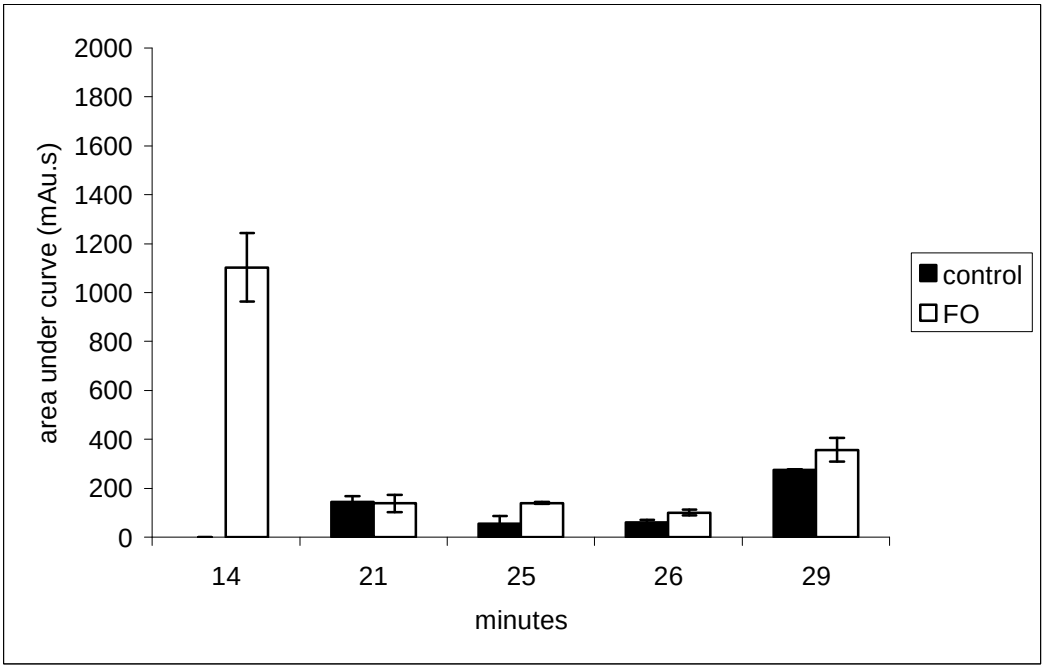

Figure.13. Accumulation of the compounds with the retention time of 14, 21, 25, 26, and 29 minutes detected at 270nm in root exudates obtained from *Fusarium oxysporum* 162 (Fo162) inoculated and non-inoculated tomato plants 3 week after fungal inoculation.

4.4. Discussion

4.4.1. Bioassay - Repellency to unconcentrated root exudates

The fact that endophytic fungi are able to produce large amounts of toxins *in vitro* indicates that they may also exude such compounds into the soil when growing *in-planta* (Sikora *et al.,* 2008). In 1996, Hallmann and Sikora reported that the secondary metabolites released from the non-pathogenic *Fusarium oxysporum* strain 162 resulted in 80% inactivation of *Meloidogyne incognita* juveniles when nematodes were exposed for 30 minutes to these metabolites. Vu (2005) investigate and tested the effects of inoculated banana with four isolates of *F. oxysporum* on the attraction of *Radopholus similis* using a linked twin-pot attraction chamber. She showed that the attractiveness of the banana roots was significantly reduced when the roots were colonized with the endophytic isolates tested. She did not determine if the effect was due to attraction or repellent activity of the roots nor did she examine if the effects were due to changes in root exudates. Similar effects on root-knot behaviour were detected in choice tests with tomato inoculated with *F. oxysporum* isolate 162 in the studies of Dababat, (2007) and Dababat and Sikora, (2007). They demonstrated that 56% of *M. incognita* juveniles failed to penetrate tomato plants pre-inoculated with the endophyte.

In the present study, an *in vitro* bioassay was used in order to determine changes in *M. incognita* behaviour induced by exposure to root exudates collected from the rhizosphere of tomato plants colonized by the same Fo162 isolate.

The results demonstrated that after 18 hours exposure, root exudates obtained from Fo162 colonized tomato plants 1, 2 or 3 weeks after inoculation, had a significant but low repellent effect on *M. incognita* J2 compared to the control exudates. These results further demonstrated that Fo162 colonization altered proprieties of host plant root exudates and exerted a certain degree of repellent activity toward root-knot juveniles *in vitro*.

4.4.2. Bioassay - Attraction to unconcentrated exudates

Because of the low level of repellency detected in the previous test, a choice bioassay test was conducted to detect the attractiveness of root exudates to root-knot J2 obtained from Fo162 inoculated plants verses the uninoculated controls. The results showed that with unconcentrated root exudates, significant differences in attractiveness between the root

exudates of Fo162 and the control were recorded within exudates collected from plants 3 weeks after Fo162 inoculation. Root exudates obtained 1 or 2 weeks after endophyte inoculation did not have a significant affect on the J2.

The lack in detection of activity of root exudates obtained from Fo162 inoculated tomato plants on the behaviour of *M. incognita* J2 with this choice bioassay could be due to the high experimental variation in the tests or to low concentrations of the substances responsible for attraction recovered from the rhizosphere soil.

Bargmann (2006) showed that the orientation of *Caenorhabditis elegans* (a mechanism called the pirouette model in *C. elegans* by Piece-Shimomura *et. al.,* 1999) is quit similar to the orientation of bacteria during chemotaxis (based random walk by Berg 1993 and Berg 1975). Pierce-Shimomura *et al.,* (2005) demonstrated that *C. elegans* can not sense a spatial gradient over its body to decide the direction to turn. Therefore, random movement behaviour of J2 under the test conditions used in the bioassay could be responsible also for lack of significant results of attraction and/or repellency of J2 to the exudates of Fo162 inoculated and non inoculated tomato plants.

Conversely, choice experiments conducted by Dababat and Sikora (2007) with sand block champers demonstrated that tomato root exudates obtained from plants colonised by Fo162 were either less attractive to *M. incognita* juveniles. They showed that 80% of root-knot nematode juveniles placed in the center of a sand-filled chamber moved away from the arm treated with root exudates obtained from tomato plants inoculated with Fo162 to the side of the chamber inoculated with root exudates of control plants.

4.4.3. Bioassay - Attraction to concentrated exudates

In order to determine if the exudates used in the above tests contained low levels of attractants due to biotic or abiotic interference a best was conducted in which the exudates were concentrated. Results of the bioassay using the root exudates concentrated through ethyl acetate extraction showed that Fo162 root exudates collected 1 or 2 weeks after fugal inoculation were more less attractive to the *M. incognita* J2 compared to the control exudates. The results illustrated that the concentrations of the chemical compounds accumulated in Fo162 root exudates play a role in the attraction of root-knot nematode and that the amounts vary between experiments. The variation in concentration probably due to levels of Fo162 colonization or to abiotic and biotic interactions present in the greenhouse.

Similar results were reported from the studies of Pierce-Shimomura *et al.,* (1999) who mentioned that *C. elegans* does not respond to the absolute level of attractants but to changes in attractants concentration over time.

4.4.4. HPLC analysis of root exudates

HPLC analysis of the biologically active compounds in root exudates of Fo162 inoculated tomato plants which may have been responsible for changes in nematode orientation behaviour was conducted. Chromatogram analysis performed at a wave length of 270nm was the most informative chromatogram and revealed differences between root exudates of Fo162 inoculated and uninoculated plants.

Results showed that there was a higher level of accumulation of compounds within the root exudates collected from Fo162 inoculated plants compared to exudates of control. The results also showed that Fo162 stimulated accumulation of a new compound which was detected at 270nm and a retention time of 14 min. The accumulation of this compound decreased in root exudates collected 2 or 3 weeks after Fo162 inoculation.

There also was a correlation between accumulation of the compounds and the biological activity of root exudates with respect to nematode behaviour. In the first experiment (bioassay-repellency, 4.2.1), the maximum accumulation of all compounds as well as the highest level of J2 repellency from Fo162 root exudates was detected within the exudates obtained from 2 week old Fo162 inoculated plants.

Similar results were obtained when the experiment was repeated (bioassays-attraction to concentrated exudates, 4.2.3). Thus the highest levels of compound accumulation and the lowest level of J2 attraction were recorded with concentred root exudates collected 1 week after Fo162 inoculation.

The analysis of the spectra patterns of the detected compounds in the exudates of Fo162 inoculated and uninoculated plants showed that the detected compounds at the same retention times were identical.

However, the chemical signals released from host plant root exudates have been shown previously to play a role in nematode infection processes.

Pline and Dusenbery (1987) demonstrated that, long distance attraction of nematodes to roots is, in general, based on non-specific chemical signals. Wuyts *et al.,* (2006) reported that plant chemicals released from roots in the rhizosphere can influence nematode behaviour. These chemicals can either attract nematodes to penetration sites or result in repellence, inhibition of mobility or even death. They also reported that the effect of plant metabolites on nematode behaviour can still be detectable after nematodes penetration.

The mechanisms by which compounds in root exudates are active against plant-parasitic nematodes are largely unknown. Hallmann and Sikora (1996) suggested that inhibitory compounds produced by the endophyte and transferred into the roots might be involved in alteration of root exudates pattern. This hypothesis has never been tested for validity.

4.5 Conclusions

Based on the present investigations the following can be concluded:

1- Root exudates of tomato plants colonized by Fo162 affected the behaviour and migration pattern of *M. incognita* J2 when nematodes were exposed for 18 hours to these exudates.
2- Low levels of repellency verses high levels of lack of attraction were detected
3- Fo162 colonization resulted in increased accumulation of several different chemical compounds in the root exudates of tomato plants.
4- One or more of these compounds could be responsible for lack of J2 attraction.
5- There is a correlation between the accumulation levels of chemical compounds in root exudates of Fo162 and biological activity of these exudates toward nematode.
6- Further studies have to be conducted to purify the detected compounds accumulating within root exudates of tomato plants colonized by Fo162.

4.6. References

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5. *Fusarium oxysporum* 162 colonization behaviour in tomato plants and its impact on the durability of induced resistance toward *Meloidogyne incognita*

5.1. Introduction

Previous studies (chapter 3) showed that the mutualistic endophyte *Fusarium oxysporum* strain 162 (Fo162) can colonize roots of Fusarium-wilt susceptible and resistant tomato cultivars. The Fo162 fungus has also been shown to induce the systemic defences of the Fusarium-wilt susceptible tomato cultivars against root-knot nematode (Dababat and Sikora, 2007a). However, in all previous studies the Fo162 stimulated systemic resistance mode of action was not determined in Fusarium-wilt resistant tomato cultivars against *Meloidogyne incognita* before.

Earlier investigations on the ability of Fo162 to colonize the plant were limited to the root system and assumed that Fo162 remains in the root systems and does not move upward into the shoots and leaves (Hallmann and Sikora 1994; Dababat and Sikora 2007). Whether or not Fo162 also colonizes aboveground stems and adventitious roots growing from the stem base and prolongs the duration of induced resistance has not been evaluated. Shoot colonization if influential in the induction process would open up additional ways of monitoring the durability of the systemic responses of endophytes such as Fo162 in plants.

Therefore in the present study, the shoots were physically detached from the roots of Fo162 pre-inoculated tomato plants. After the newly formed adventitious roots had developed, the presence of Fo162 inside the shoots and the roots was determined and the colonization of *M. incognita* in these newly formed adventitious roots was followed over time.

The stem detachment technique which was used in this study was used before by Fuchs *et al.,* (1997) to determine the ability of another non pathogenic *Fusarium oxysporum* strain (Fo47) to induce resistance to the Fusarium-wilt pathogen in tomato plants. They reported that the initial inoculation of Fo47 into rock wool, which was used to grow the tomato plants before detachment, reduced the incidence of Fusarium wilt from 38 to 20% in the adventitious roots of the tomato stems transplanted later into potting mix inoculated

with the pathogenic isolate of *F. oxysporum* (Fo18) .They also reported that similar results were obtained with three different bioassays which were conducted to investigate whether the non pathogenic isolate Fo47 was able to induce resistance against an isolate of the Fusarium wilt pathogen (Fo18) when they were introduced separately in time to the challenge. However the analysis did not determine the presence of Fo47 inside the plants, nor did they consider wound induced responses.

The objectives of this study were to determine the:

1- Capability of Fo162 to colonize the shoots and the newly formed shoot adventitious roots of the primary Fo162 inoculated tomato cultivars.
2- Ability of Fo162 to induce the systemic defences in detached shoots of Fusarium-wilt susceptible and resistant tomato cultivars against *M. incognita*.
3- Durability of the signals related to the mutualistic endophyte *F. oxysporum* (Fo162) systemic resistance in detached shoots of both tomato cultivars.

5.2. Materials and methods

5.2.1. Shoot detachment and initial Fo162 root colonization

Seeds of two tomato cultivars, (P48027, India) susceptible and (SUNGUARD, XP 10089–Thailand) resistant to Fusarium-wilt, were sown separately in plastic pots filled with an autoclaved soil:sand mixture (1:2 v/v).

Two weeks after sowing, each tomato seedling was inoculated with 10^6 cfu/g soil of Fo162 while the control plants were treated only with tap water. Two weeks after Fo162 inoculation, the root systems of all tomato seedlings were cut-off and the detached shoots were replanted in new pots filled with the autoclaved soil:sand substrate. The root systems were used to verify the colonization levels of Fo162. Colonization tests were performed as described in chapter 3 (3.2.1).

5.2.2. Colonization of Fo162 in shoots and newly formed shoot adventitious roots

Shoots and the adventitious stem roots were verified for the presence of Fo162. The basal ends of the shoots (3 cm above soil surface) and the newly produced adventitious roots were surface sterilized and monitored on PDA plates for endophytic growth (as mentioned in chapter three, (3.2.1), (Fig.1). The emerging fungal isolates were sub-cultured on PDA and morphologically and microscopically characterized (Fig.2). The isolates which resembled Fo162 were further characterized by RFLP analysis of the IGS regions.

Figure.1. Isolation of *Fusarium oxysporum* 162 from the newly formed adventitious roots growing out of the transplanted detached shoots of tomato following soil inoculated with Fo162 (right plate) and control plants (left plate).

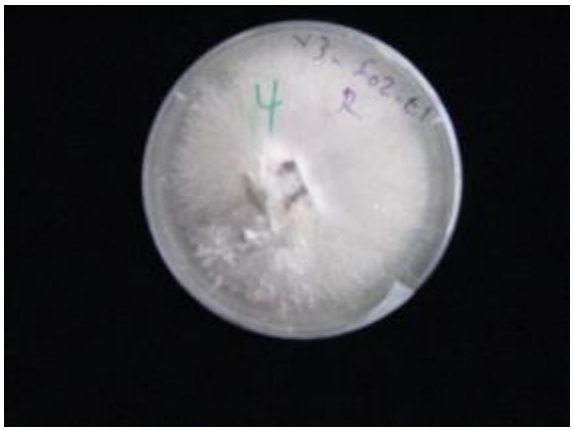

Figure.2. Pure cultures of the endophytic fungi their culture characteristic corresponded to *Fusarium oxysporum* 162.

5.2.3. IGS- RFLP analysis

Fungal isolates which had culture and microscopic characteristics corresponding to those of Fo162 were further characterized by restriction fragment length polymorphism (RFLP) analysis of the ribosomal intergenic spacer (IGS) regions as described by Edel *et al.,* (1995 and 1997). IGS PCR fragments were amplified by PCR using the primers PNFo (5`-CCCGCCTGGCTGCGTCCGACTC -3´), and PN22 (5`- CAAGCATATGACTACTACTGGC-3´) and subsequently digested with 3 restriction enzymes: *Alu*I, *Hae*III and *Rsa*I. Each isolate was assigned to an IGS type, defined by the specific restriction patters obtained with the three enzymes. The pairwise restriction site differences between IGS types were represented as a dendrogram with the computer program using NTEdit and NTSYSpc Numerical Taxonomy System, Version 2.2 (Exeter software) as described in chapter 2.

5.2.4. Durability of systemic resistance signals in detached shoots and adventitious roots

In order to determine the durability of the signals of Fo162 induced resistance in tomato plants toward the root-knot nematode a detached shoot bioassay was developed. The hypothesis was that once the signals moved from the original root system into the shoot, the shoot of the plant would retain the ability to induce resistance basal pedal into the newly formed adventitious root system as seen in split-root tests (Dababat and Sikora 2007). Therefore, the detached shoots of 6 plants, which were initially inoculated with Fo162 and 6 plants of the non-inoculated control, were inoculated separately with 1000 juveniles (J2) of *M. incognita* (as mentioned in chapter 2). The adventitious roots were inoculated with J2, 5, 7 or 10 days after transplanting the detached shoots into the autoclaved soil.

The plants developing out of the stems were grown under greenhouse conditions at 25 ± 5 °C and fertilized by NPK (14:10:14) with concentration of 3g/L one time per week.

Two weeks after nematode inoculation, the tomato plants were removed from the soil and the newly formed adventitious roots were washed gently with tap water and blotted between tissue paper. The root weight was recorded and the number of galls was counted for each tomato plant.

5.3. Results

5.3.1. Shoot detachment and initial Fo162 root colonization

Results showed that the mutualistic endophyte *F. oxysporum* (Fo162) successfully colonized the root systems of the non-detached Fusarium-wilt susceptible and resistant tomato cultivars. No differences in Fo162 colonization levels were detected between the two cultivars. Furthermore, the percentage of Fo162 colonization reached approx. 85% on both cultivars (Fig.3).

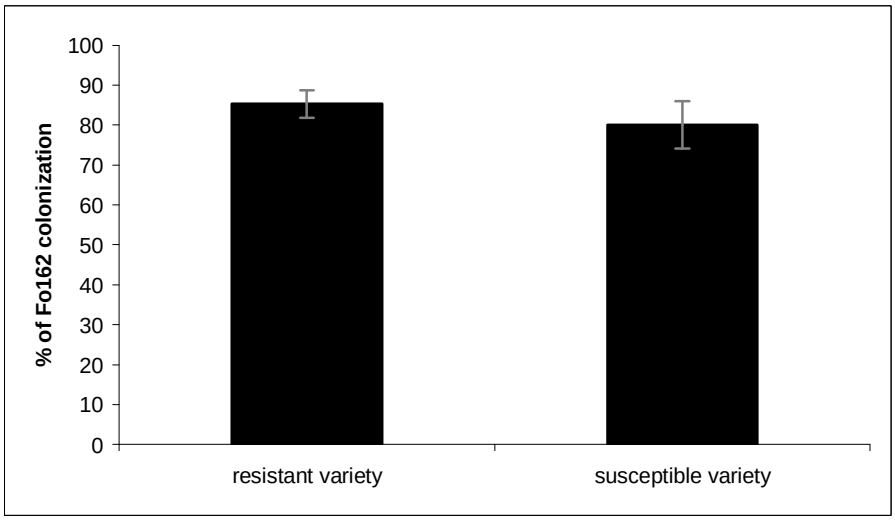

Figure.3. *Fusarium oxysporum* 162 colonization levels in the initially inoculated roots of the Fusarium-wilt susceptible (P48027, India) and resistant (SUNGUARD, XP 10089–Thailand) tomato cultivars. Means pared with (*) are significantly different based on Tukey test (*p* 0.05; n = 6).

5.3.2. Colonization of Fo162 in shoots and newly formed shoot adventitious roots

Fo162 was identified using the morphological and microscopic observations of the fungal isolates that colonized the stem base and newly formed shoot adventitious roots. The results showed that 7 days after detachment, Fo162 successfully colonized the shoots basal ends and the newly formed shoot adventitious roots. Approximately 20 % of the Fo162 shoots of both cultivars, 3cm above soil surface, were found to be colonized by the endophyte.

The level of colonization of the newly formed stem adventitious roots also reached 30% within the Fusarium-wilt resistant and the susceptible tomato cultivars tested, (Fig.4).

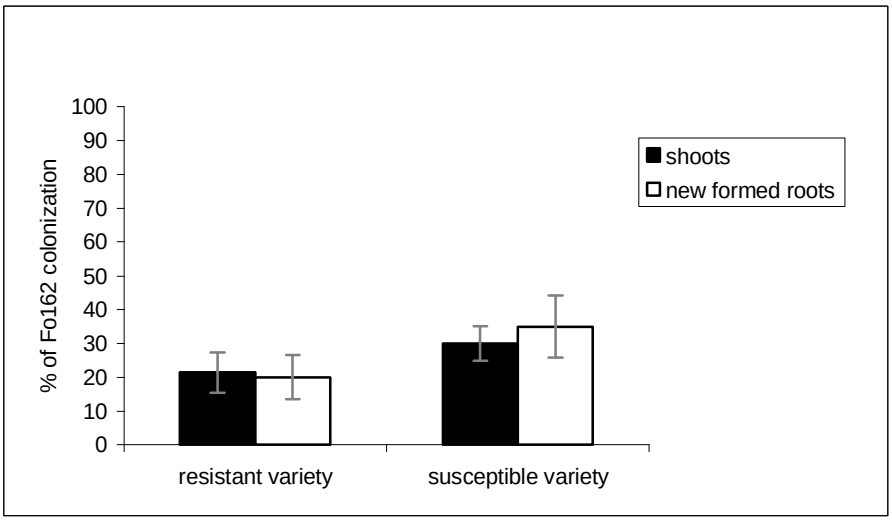

Figure.4. Percentage of *Fusarium oxysporum* 162 colonization in the shoots basal ends and the newly formed shoot adventitious roots of the Fusarium-wilt resistant and susceptible tomato cultivars 7 days after detachment from the Fo162 pre-inoculated plants. Means pared with (*) are significantly different based on Tukey test (*p* 0.05; n = 6).

Similar results were observed with the Fusarium-wilt resistant tomato plants 10 days after stem detachment from the Fo162 primary inoculated root systems. However, whereas Fo162 colonization reached 20% within the shoots ends and the newly formed shoot adventitious roots of the Fusarium-wilt resistant tomato cultivar, it reached 50% within the newly formed stem based adventitious roots of the Fusarium-wilt susceptible plants, (Fig.5).

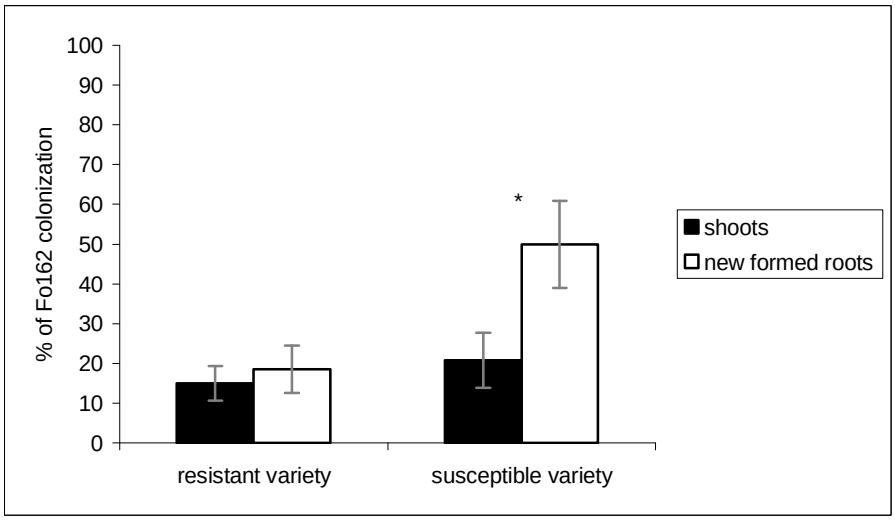

Figure.5. Percentage of *Fusarium oxysporum* 162 colonization in the shoots basal ends and the newly formed shoot adventitious roots of the Fusarium-wilt resistant and susceptible tomato cultivars 10 days after detachment from the Fo162 pre-inoculated plants. Means pared with (*) are significantly different based on Tukey test (*p* 0.05; n = 6).

5.3.3. IGS-RFLP analysis

To confirm the identification of the isolates which had growth and microscopic characteristics corresponding to those of Fo162 (5.3.2.), restriction fragment length polymorphism (RFLP) analysis of the IGS PCR amplified fragments was carried out with 17 isolates obtained from the basal ends of the shoots and from the newly formed shoot adventitious root segments, (Fig.6).

The results obtained verified that the genotype of only 4 isolates was identical with the Fo162 genotype. The homogeneity between the rest of the isolates and Fo162 ranged from 69 to 85%, (Fig.7).

These results revealed that not all fungal isolates that have been identified, in the 5.3.2. colonization test, as Fo162 according to the morphological and microscopic examination, represented the Fo162 endophyte fungal.

Therefore, the morphological and microscopically identification of those isolates as Fo162 was partially consistent with the molecular identification.

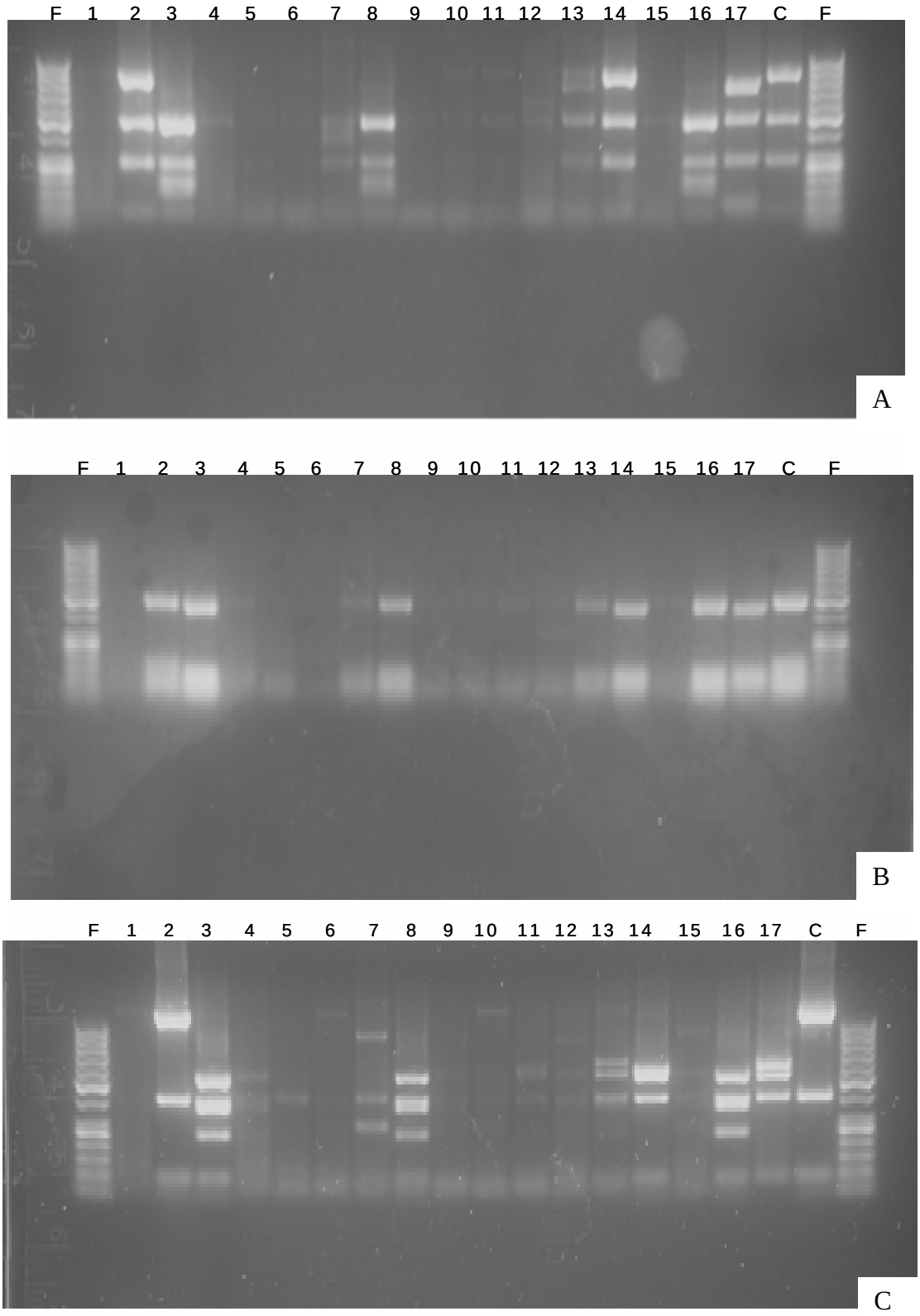

Figure.6. The restriction patterns of 17 isolates of the fungi isolated from the detached shoots and the newly formed shoot adventitious roots of the *Fusarium oxysporum* 162 primary inoculated plants, revealed with three restriction enzymes, *Alu*I (A), *Hae*III (B) and *Rsa*I (C) for IGS type.
F= 50bp ladder. C= Fo162 reference.

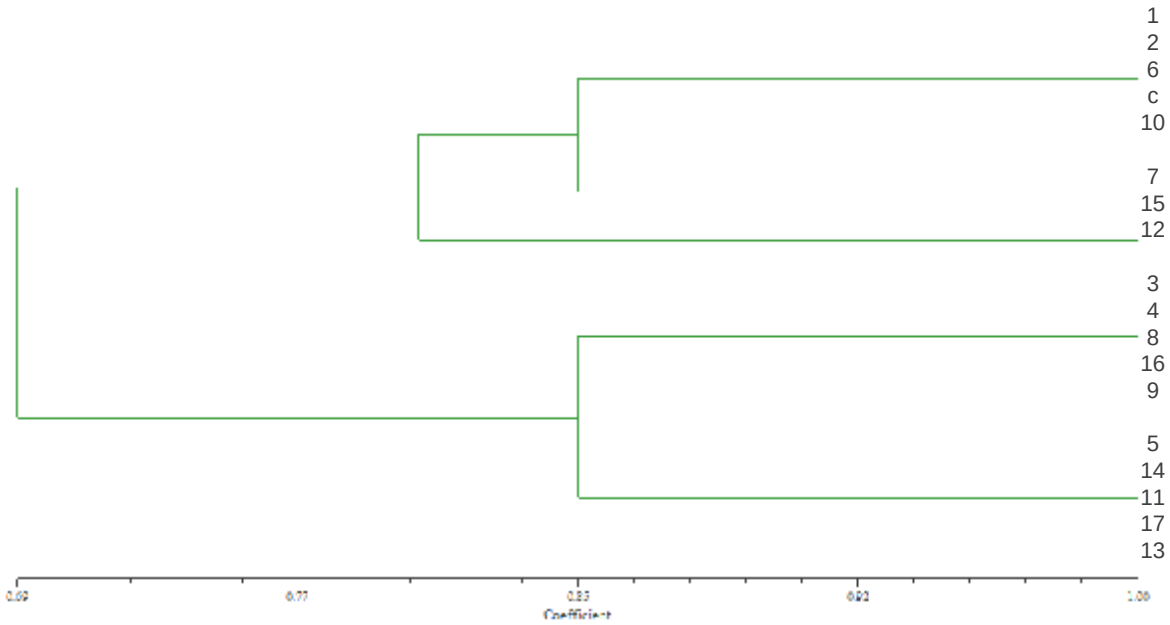

Figure.7. Dendrogram of 17 isolates of the fungi isolated from the detached shoots and the newly formed shoot adventitious roots of *Fusarium oxysporum* 162 primary inoculated plants based on the restriction analysis of the IGS. Bars indicate genetic the similarity [%] amongst taxa based on the Dice coefficient of similarity. C= Fo162.

5.3.4. Durability of systemic resistance signals in detached shoots and adventitious roots

The results showed that the adventitious roots of the tomato plants which were inoculated with the *M. incognita* J2 five days after detachment from the Fo162 inoculated plants had significantly lower numbers of galls/g root. The reduction in nematode gall number was approximately 51 % between the control plants and the Fo162 pre-inoculated plants on the Fusarium-wilt resistant and susceptible cultivars, (Fig.8).

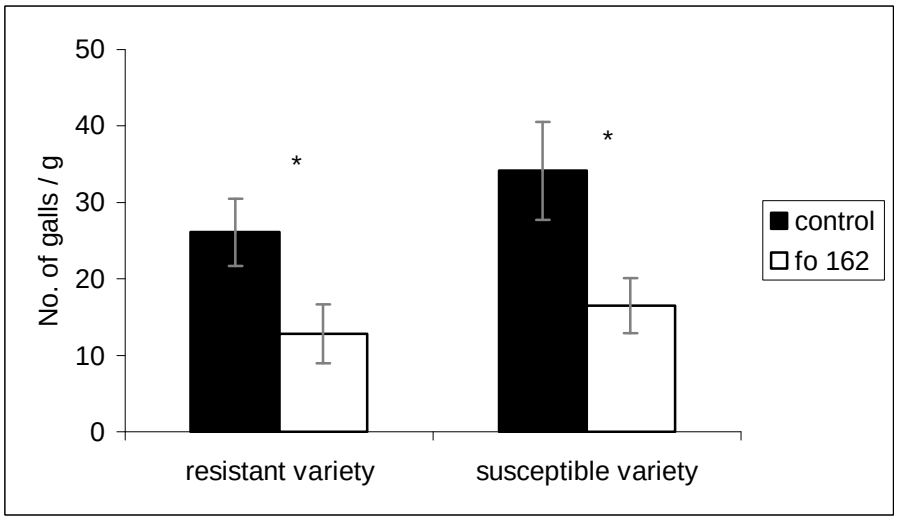

Figure.8. Number of galls/g root recorded on the Fusarium-wilt resistant and susceptible tomato adventitious roots inoculated with *Meloidogyne incognita* 5 days after stem detachment from the *Fusarium oxysporum* 162 pre-inoculated plants. Means pared with (*) are significantly different based on Tukey test (*p* 0.05; n=6).

Similar results were observed again within tomato plants which were inoculated with *M. incognita* 7 days after detaching the Fo162 inoculated primary root systems. The reduction in nematode galls number reached up to 43 and 49% on the Fusarium-wilt resistant and susceptible tomato cultivars, respectively, (Fig.9).

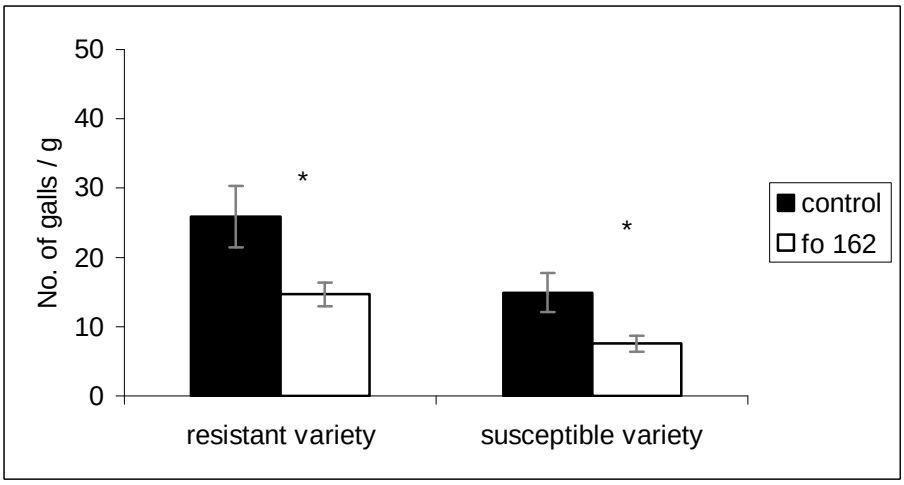

Figure.9. Number of galls/g root recorded on the Fusarium-wilt resistant and susceptible tomato cultivars inoculated with *Meloidogyne incognita* 7 days after separating the *Fusarium oxysporum* 162 primary inoculated roots. Means pared with (*) are significantly different based on Tukey test (*p* 0.05; n=6).

The results also demonstrated that 10 days after stem detachment of the Fo162 inoculated tomato plants, the systemic resistance signals no longer affected nematode infection on the two cultivars. There were no significant differences in number of galls on the newly formed adventitious stem roots between the control plants and those initially inoculated with Fo162, (Fig.10).

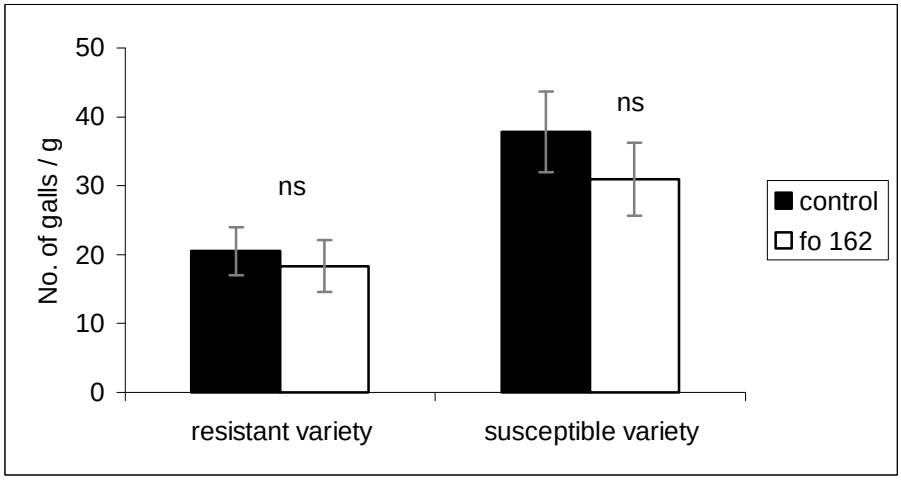

Figure.10. Number of galls/g root recorded on the Fusarium-wilt resistant and susceptible tomato cultivars inoculated with *Meloidogyne incognita* 10 days after separating the *Fusarium oxysporum* 162 primary inoculated roots. Means pared with (*) are significantly different based on Tukey test (*p* 0.05; n=6).

5.4. Discussion

5.4.1. Shoot detachment and initial Fo162 root colonization

The results of the presented study showed that Fo162 successfully colonized the primary inoculated roots of both Fusarium-wilt susceptible and resistant tomato cultivars to high levels. Two weeks after Fo162 inoculation, root colonization of the endophyte reached 80% within both two tomato cultivars. Similar results were reported in the previous study (chapter 3). Furthermore, Dababat *et al.,* (2008) also reported that the mean level of Fo162 colonization ranged between 32 and 100% among the different tomato cultivars they tested. They also reported a positive correlation between Fo162 colonization levels and *M. incognita* biological control on tomato plants.

Colonization levels of fungal endophytes have been shown to play a crucial role in the antagonistic activity against plant parasitic nematodes. Speijer (1993) and Hallmann and Sikora (1994a,b) reported that colonization of the endorhiza is important for self propagation of the endophyte, for biological control and for the accumulation of endophytic secondary metabolites and toxic compounds that may be important in enhancement of plant defences mechanisms in the root tissues.

5.4.2. Colonization of Fo162 in shoots and newly formed shoot adventitious roots

Based on the morphological and microscopic identifications, the endophyte fungus Fo162 was shown to be able to colonize both the detached shoot basal ends and the newly formed adventitious roots of the pre-inoculated plants.

The Fo162 colonization levels in the detached shoots and the newly formed adventitious roots were lower (Fig.4 and Fig.5) compared to the colonization levels observed in the root systems of the pre-inoculated plants before detachment (Fig.3). These results indicated that Fo162 may grow systemically from intact pre-inoculated tomato roots into the stem base and from there into the adventitious root systems which are initiated later at the base of the detached shoots. At the same time, the presence of Fo162 in the detached shoots and the new formed roots segments could support induce resistance durability by prolonging single induction processes. This is the first report of the systemic growth of Fo162 into the stem base and shoots adventitious root system.

5.4.3. IGS-RFLP analysis

To confirm the results obtained from the morphological and microscopic investigations (5.3.2.), molecular identification of the fungal isolates which resembled Fo162 was performed using restriction fragment length polymorphism (RFLP) of the ribosomal intergenic spacer (IGS) PCR amplified fragments.

The data showed that the results of the morphological and microscopic investigations were not always consistent with the results of the molecular investigation. Of the 17 isolates tested in this investigation, the genotype of only 4 isolates could be verified as identical with the Fo162 genotype whereas the other isolates had close similarity to Fo162. The level of similarity ranged from 69 to 85%. These results illustrated that other non-pathogenic isolates in the potting soil or water also colonized the shoot basal ends and the newly formed adventitious roots.

The results obtained from the molecular identification indicated also that the morphological and microscopic observations are not foolproof and can not be relied upon to give 100 percent characterization of the presence of Fo162 isolates in pre-inoculated plants.

The molecular technique (IGS-RFLP) which was used in this study to verify the presence of Fo162 isolates was used also by Zambounis *et al.,* (2007). They also used the polymerase chain reaction (PCR) amplification of the ribosomal intergenic spacer (IGS) regions combined with digestion with three restriction enzymes (*Alu*I, *Hae*III, *Rsa*I) technique to identify and distinguish the isolates of an Australian *Fusarium oxysporum* f. sp*. vasinfectum* among various isolates belonging to *Fusarium oxysporum*. Furthermore, Lori *et al.,* (2004) used the intergenic spacer (IGS) typing procedure to study the genetic diversity among 151 isolates of *Fusarium oxysporum* that belonged to pathogenic and non-pathogenic Fusarium populations isolated from carnation fields in Argentina. They reported that IGS typing is a useful tool in the identification of the *Fusarium oxysporum* forma specialis.

5.4.4. Durability of systemic resistance signals in detached shoots and adventitious roots

The shoot detachment technique was used in this study to investigate the durability of Fo162 induced systemic defences signals toward *M. incognita* in the Fusarium-wilt susceptible and resistant tomato cultivars.

The results from these investigations showed that *M. incognita* infection was significantly reduced on detached adventitious roots 5 and 7 days after detachment from the Fo162 pre-inoculated primary root systems. Five days after separation from the Fo162 inoculated primary roots, the reduction in number of galls/g reached 50 and 51% on the Fusarium-wilt resistant and susceptible tomato cultivars, respectively.

Similar results were observed when *M. incognita* juveniles were inoculated 7 days after detaching the Fo162 inoculated primary roots. The reduction in number of galls/g reached 43 and 49% within the resistant and susceptible tomato cultivars, respectively.
Conversely, when nematodes were inoculated 10 days after detachment, no significant differences in the number of *M. incognita* galls were detected between the detached stems from the Fo162 inoculated plants and the non-inoculated control plants.

The results seem to indicate that the signals of Fo162 based systemic resistance in tomato plants together with the additive effects of wound induced defences, which could have been elicited when the shoots were detached from the pre-inoculated roots, were responsible for the reduction in number of galls observed on the Fo162 shoots 5 and 7 days after detachment. Induced systemic resistance (ISR) has been shown to be wound inducible thus wounds can elicit the ISR pathways in different host plants (Bostock *et al.,* 2001).

Other researchers have demonstrated that different strains of endophytic fungi can protect plants through different mechanisms of action and that induced resistance is considered important as a triggering factor in the interactions between endophytes, host plants and plant parasites (Dababat and Sikora, 2007a,b; Cramer *et al.,* 1985; Biles and Martyn, 1989). Matta (1989) reported that induced resistance can be either local (associated with a hypersensitive reaction) or systemic (provides an entire host plant protection against later pathogens infections).

In 1989, Strömberg mentioned also that systemic induced resistance can be obtained when challenge inoculum is applied at a later time and different location on a plant.

Similar results to the obtained results from the present study were obtained by Hallmann and Sikora (1994a); Dababat and Sikora (2007b); Dababat *et al.* (2008), who reported that the non-pathogenic *Fusarium oxysporum* strain (Fo162) had a high level of biological control potential in reducing nematode infection on tomato. Dababat and Sikora (2007a) investigated the mode of action of Fo162 in tomato plants and demonstrated that Fo162 was capable of protecting tomato from root-knot nematode infection by inducing host plant systemic resistance and by triggering other mechanisms of action.

Two new findings were made in the present investigations: 1) that Fo162 has the ability to induce systemic resistance to the root-knot nematode in tomato plants that are resistant to Fusarium-wilt and 2) that the durability of the signals related to the Fo162 systemic resistance is emitted from the stem into the adventitious roots after detachment from the initial induced root system of the mother plants.

5.5. Conclusion

Based on the present investigation the following can be concluded:

1- Fo162 was able to colonize with high levels, roots of the Fusarium-wilt susceptible and resistant tomato cultivars.
2- Fo162 was able to induce the systemic defences toward *M. incognita* in Fusarium-wilt susceptible and resistant tomato cultivars when the endophyte was not in the direct contact with the host plant or with the nematode.
3- The Fo162 signals responsible for systemic induced resistance were responsible for protecting tomato plants from root-knot nematode invasion for up to 7 days after the physical separation between the host plant and the endophyte fungus. This indicates that the Fo162 signals that are based in the shoot also add to durability of the control mechanism.

5.6. References

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6. Induction of systemic resistance in tomato toward the root-knot nematode *Meloidogyne incognita* using biotic and abiotic elicitors

6.1. Introduction

Various mutualistic endophytes can serve as antagonists against a wide range of plant pathogenic fungi and nematodes. In such interactions, different modes of action could be involved in the control of the pathogen and induced resistance in the host, elicited by the endophyte, is considered one of them (Sikora *et al.,* 2007).

Different types of systemic resistance can be induced within host plants either biologically or chemically using different kinds of biological and chemical elicitors (Heil and Bostock, 2002; Pieterse *et al.,* 2001; and Walling, 2000).

Biologically induced resistance in host plants can be obtained using biotic elicitors e.g. viruses, bacteria and fungi. Leemann *et al.*, (1995) and Zhou and Paulitz (1994), for example, demonstrated the ability of *Pseudomonas* bacteria to induce plant systemic resistance against Fusarium wilt and *Pythium aphanidermatum* pathogens, respectively.

Hasky *et al.,* (1998) were able to induce system resistance to the potato cyst nematode *Globodera pallida* using the endophytic bacteria *Agrobacterium radiobacter* (G12). In split root experiments with tomato plants where one half of the roots was treated with cell suspension of rhizobacteria and the other side was inoculated with *Meloidogyne javanica*, Siddiqui and Shaukat (2004), showed that the nematode population in the treated plants was reduced significantly compared to the nematode population in the non-treated tomato plants.

Induction of systemic defences against various disease organisms can also be obtained through the application of chemical elicitors which trigger different signalling pathways. Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR) are the two main resistance pathways which are mediated through the jasmonic acid metabolic signalling pathway or the salicylic acid signalling pathway, respectively (Sticher *et al.,* 1997; and Ton *et al.,* 2002).

In this study, systemic resistance in tomato induced by the fungal endophyte Fo162 was compared with induced systemic resistance (ISR) and systemic acquired resistance (SAR) that are chemically induced using methyl jasmonate (MJ) and salicylic acid (SA), respectively.

The effects of systemic resistance on nematode infection and egg masses production of the sedentary nematode *M. incognita* as well as on the physiology of tomato plants were monitored using the split-root experimental design.

The objectives of this study were to determine the:

1- Ability of different biological (Fo162) and chemical (SA and MJ) elicitors to induce systemic resistance in tomato plants against root-knot nematodes.

2- The influence of inducing host plant systemic resistance using biological (Fo162) and chemical (SA and MJ) elicitors on plant growth and physiological properties of tomato.

6.2. Materials and methods:

6.2.1. Root growth and nematode infection

The ability of biotic and abiotic elicitors to induce systemic resistance in tomato against *M. incognita* was investigated using the mutualistic endophyte *Fusarium oxysporum* strain 162 (Fo162), salicylic acid (SA) and methyl jasmonate (MJ). Seeds of the tomato cultivar ´´Hellfrucht-Frühstamm``, which is susceptible to *M. incognita*, were sown separately in plastic pots containing an autoclaved soil:sand mixture (1:2 v/v). Three weeks after sowing, or when the seedlings were about 25-30 cm in height, the shoots were cut-off 5 cm from the basal ends. The shoots were then longitudinally split into 2 halves and each half was replanted in new substrate in two neighbouring pots. One week after splitting and replanting the shoots, new adventitious roots developed in each pot. At this time one pot of the twin pot set was inoculated with either a biological or a chemical inducer. This pot was termed the inducer side, while the other pot was termed the responder side, (Fig.1).

To evaluate the influence of the biological and chemical inducers on nematode infection, the inducer sides of 6 plants were inoculated with 10^6 cfu/g soil of Fo162 (as described in chapter 2), or treated with 200 µM SA, 100 µM MJ, 10 µM MJ, or with tap water (control). One week after treating the inducer sides with the elicitors, the roots at the responder side were inoculated with 450 *M. incognita* juveniles (as described in chapter 2). The plants treated with the chemical inducers at the inducer side were watered daily with the respective inducing solutions. The control plants and the Fo162 inoculated plants were watered daily with tap water.

Stock solutions of salicylic acid and methyl jasmonate substrates were prepared with concentrations of 20 mM and 10 mM respectively by adding the SA or MJ to water followed by shaking for 1 h on a magnetic stirrer. These stock solutions were stored in the dark and used for preparing the desired concentrations immediately prior to watering. All chemicals were purchased from Sigma-Aldrich. Tomato plants were grown under greenhouse conditions at 25 ± 5 °C with 16 h diurnal light and fertilized once a week with 2g/L NPK (14:10:14).

The experiment was terminated 3 weeks after nematode inoculation. Roots of the responder side and the inducer side were washed gently with tap water then blotted between tissue paper. The fresh root weights were recorded and the number of galls per root system of the responder sides was counted.

The experiment was repeated twice using the same procedures mentioned above. However, in the second repetition, the number of egg masses in addition to the number of galls on the root systems at responder sides was determined. The egg masses on the roots were stained with 0.015% Phloxine B for 20 minutes and washed with tap water to remove excess staining solution. This experiment was terminated 4 weeks after nematode inoculation.

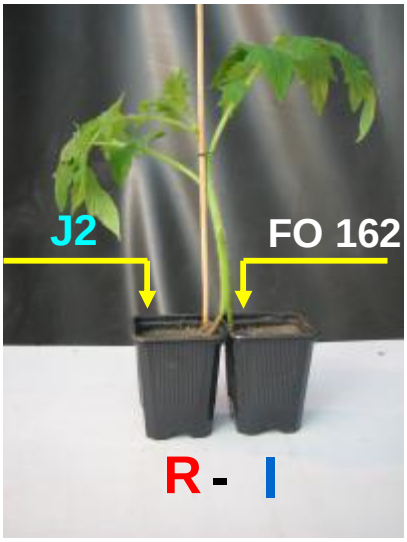

Figure.1. Split-root system bioassay used in evaluating the influence of the application of the biotic elicitor *Fusarium oxysporum* 162 or the abiotic elicitors salicylic acid and methyl jasmonate on *Meloidogyne incognita* infection and root growth in tomato plants.
J2 = root-knot juveniles; R= responder side and I= inducer side.

6.2.2. Plant transpiration

To determine plants transpiration levels and or water stress, of the tomato plants, IR-thermal images that record plant temperature were recorded. The digital images were obtained using a Varioscan 3201 ST camera (Jenoptic Laser, Jena, Germany). Data were gathered 14, 21 and 28 days post nematode inoculation. Before measuring shoot temperature, the tomato plants were kept in darkness for 1 h to let the plants acclimate. IR-Thermometric measurements were conducted from 7:00 to 9:00 am in darkness. In general, the temperature in the chamber was 24±3 °C with a relative humidity of 60±10 %. To compensate for variations, plant temperatures were measured randomly. The statistical analysis was done with SPSS. The data was analyzed using Tukey test at a probability level 0.05.

6.2.3. Chlorophyll content

The same plants used for measuring temperatures (6.2.2), were used to determine leaf chlorophyll content. Three leaves of each individual plant were measured 14, 21 and 28 days after nematode inoculation using the chlorophyll meter (SPAD-502, Minolta, Japan) and five measurements were taken for each leaf. The average of those fifteen measurements for each plant was calculated.

6.3. Results

6.3.1. Root growth at inducer side

The influence of the elicitors on the root growth at the inducer side of the treated plants was determined. The results of trial A showed that 100 µM MJ inhibited significantly the root growth at the inducer sides when compared to the control and to the other elicitors (Fo162 and SA), (Fig.2.A). Fo162 and SA had no significant effects on root growth at the inducer side of treated plants.

In trial B, the root growth at the inducer side of split-root tomato plants treated with the SA or MJ was significantly reduced when compared to the control. The biotic elicitor, Fo162, did not affect root growth at the inducer side of inoculated plants, (Fig.2.B).

Trial C showed that all tested three chemical elicitor, 200 µM SA, 100 µM and 10 µM MJ, reduced root weight at the inducer side of split-root tomato plants. Conversely, the biological inducer, Fo162, did not affect root growth when compared to the control, (Fig.2.C).

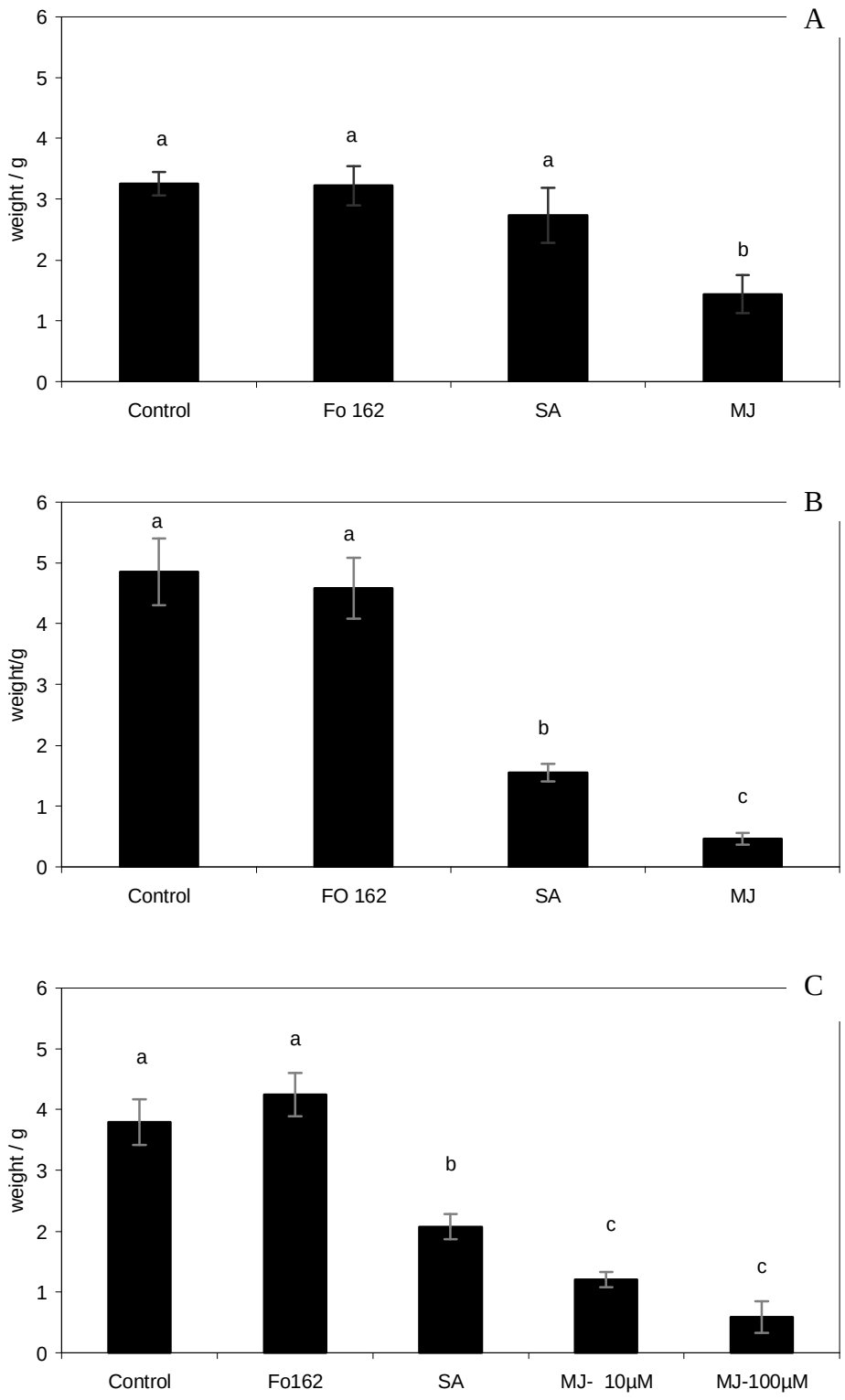

Figure.2. Effects of *Fusarium oxysporum* (Fo162), methyl jasmonate (MJ) and salicylic acid (SA) elicitors on root weight at the inducer side of a split-root tomato plant obtained in 3 separate experiments A, B and C. Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

6.3.2. Root growth at responder side

At the responder side, no significant differences in root weight were recorded between the control and the plants treated with the biotic and abiotic elicitors, in trial A, (Fig.3.A).

Similar results for root growth were observed for all treatments in trial B, (Fig.3.B). However, slight increases in root weight were observed at the responder side of Fo162 inoculated tomato plants.

In trial C, only 100 µM of methyl jasmonate significantly reduced root weight at the responder side when compared to the control. The other elicitors (Fo162, 200 µM SA and 10 µM MJ) showed no reduction in root growth, (Fig.3.C).

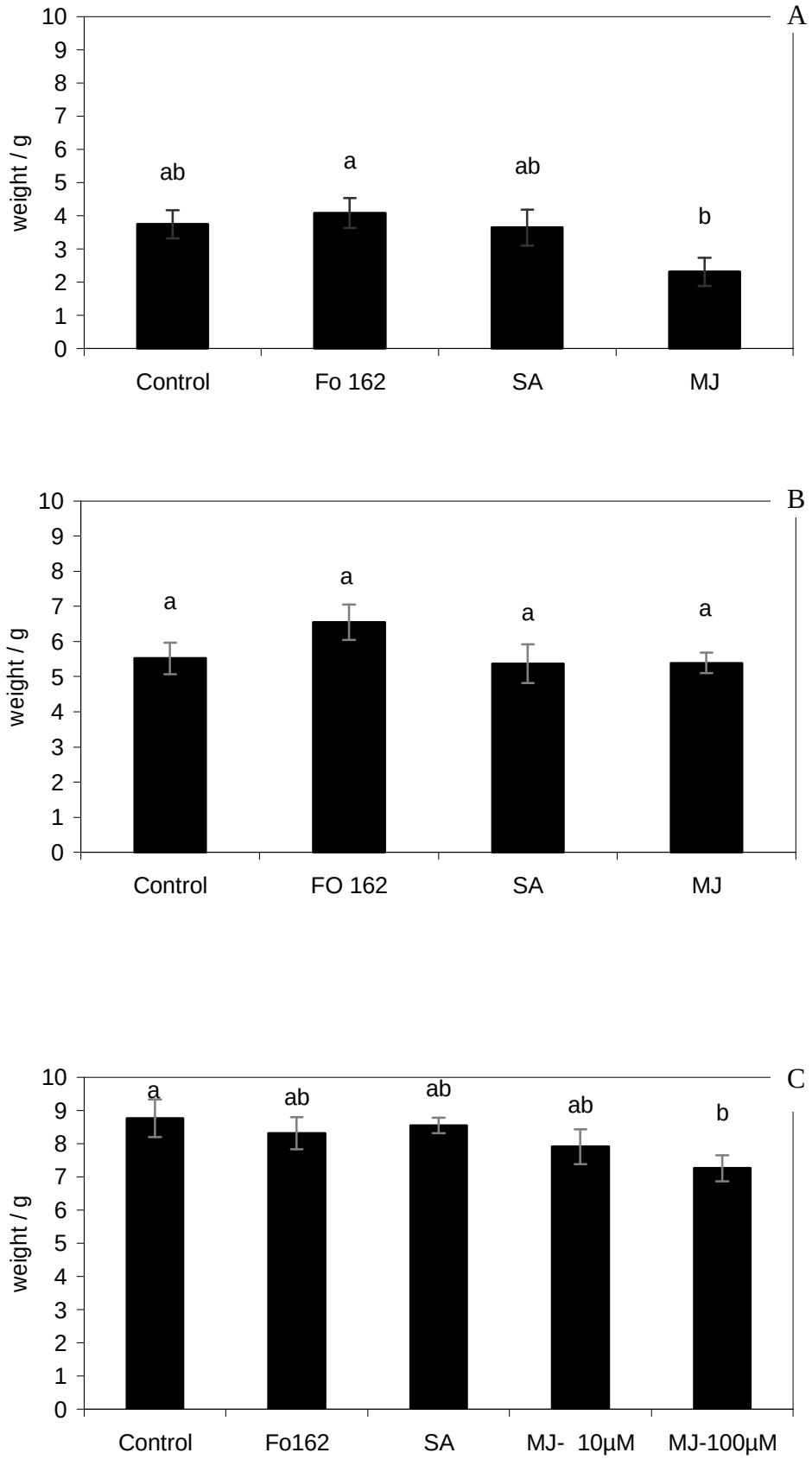

Figure.3. Effects of *Fusarium oxysporum* (Fo162), methyl jasmonate (MJ) and salicylic acid (SA) elicitors on root growth at the responder side of a split-root tomato plant obtained in 3 separate experiments A, B and C. Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

6.3.3. Nematode infection

In trial A, the results showed that the biological, Fo162, and the chemical elicitors, 200 µM SA and 100µM MJ, inhibited *M. incognita* infection on the responder side of the split-root tomato plants. The total number of galls per root system as well as the number of galls/g root was significantly reduced at the responder side of the tomato plants treated with all three elicitors when compared to the control, (Fig.4 and 5). The reduction in nematode galls reached approximately 67, 75, and 91% in the Fo162, SA and MJ, treated plants, respectively.

In trial B, the results showed that both the biological, Fo162, or the chemical, SA and MJ, elicitors significantly reduced the number of galls/root system, (Fig.6) and the number of galls/g root, (Fig.7). The reduction in nematode galls reached approximately 22, 40 and 79% in the Fo162, SA and MJ, treated plants, respectively.

In trial C, the results showed also that Fo162, 200 µM SA and 100 µM MJ significantly reduced the number of galls per root system compared to the control. The highest reduction in number of galls (41%) was observed for the plants treated with100µM MJ followed by Fo162 (27%) and 200µM SA (24%), (Fig.8).

Similar results were obtained in trial (C) when the number of galls/ g root was determined on roots of elicitor treated verses non treated plants, (Fig.9).

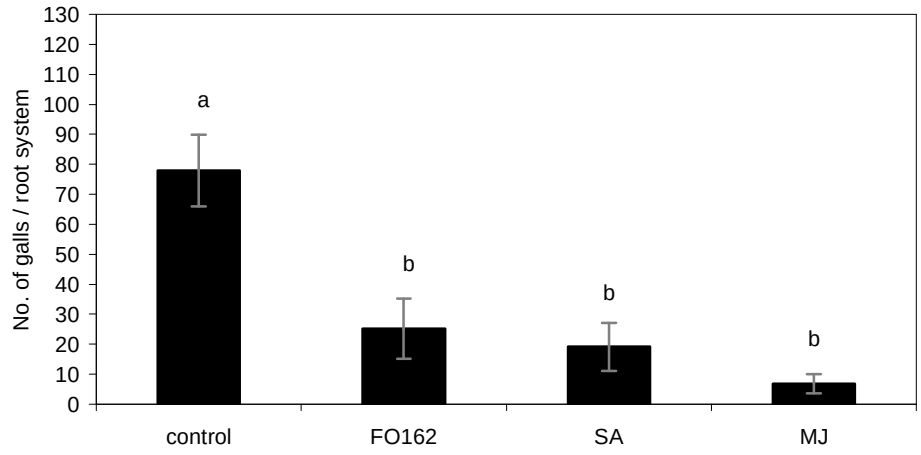

Figure.4. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on nematode galls / root system at the responded side of a split-root tomato plant (trial A). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

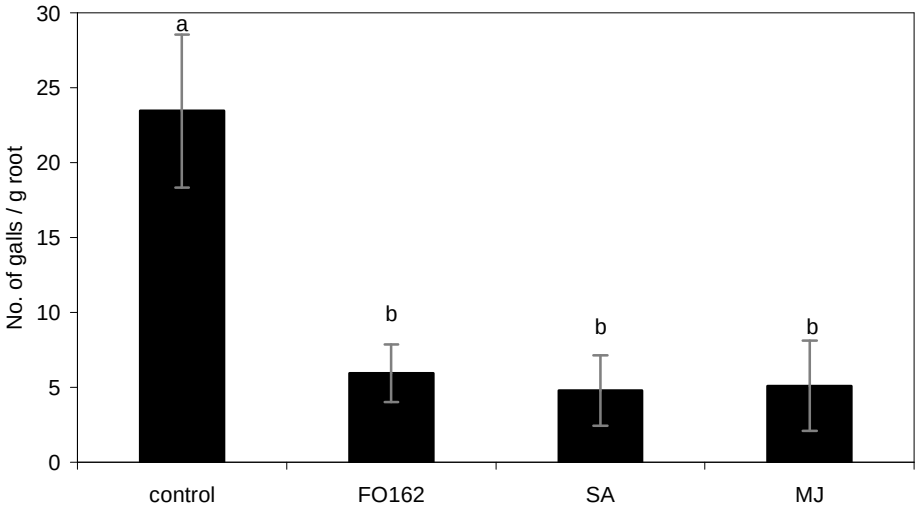

Figure.5. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on nematode galls / g root at the responded side of a split-root tomato plant (trial A). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

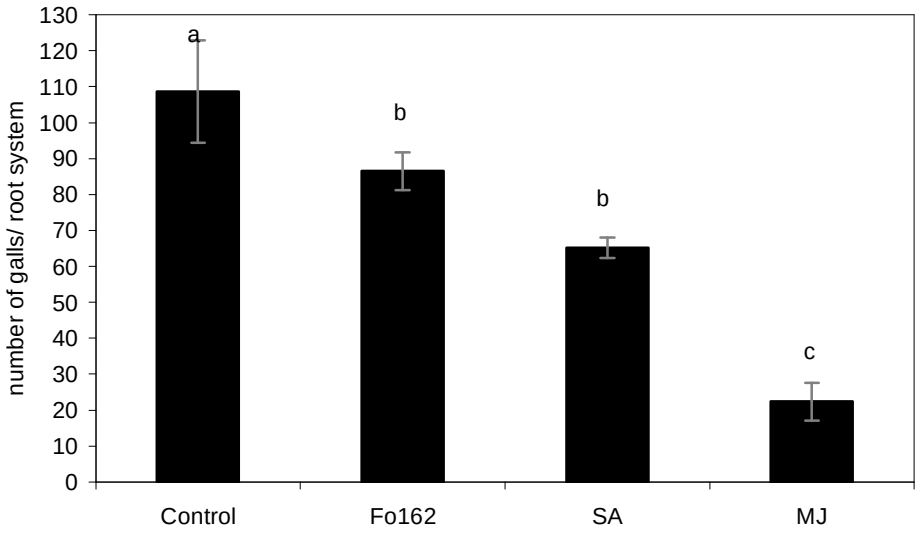

Figure.6. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on nematode galls / root system at the responded side of a split-root tomato plant (trial B). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

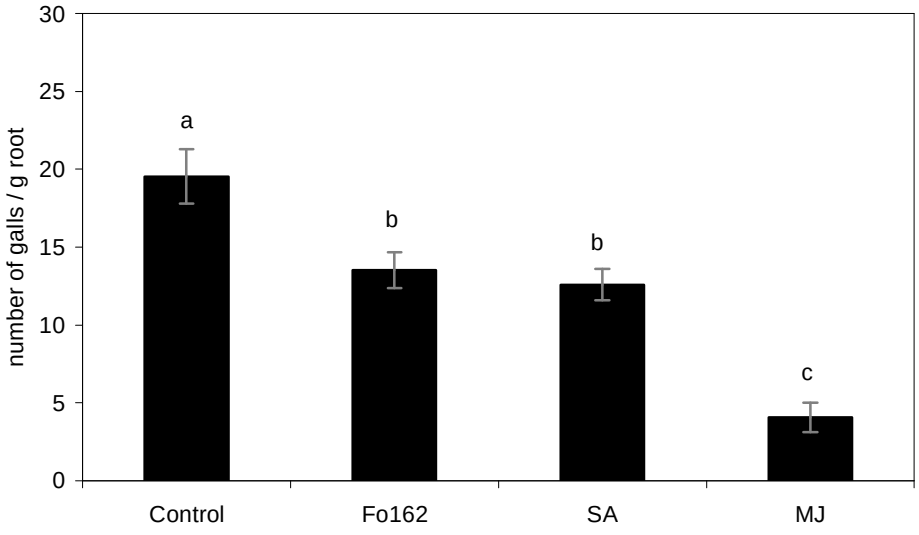

Figure.7. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on nematode galls / g root at the responded side of a split-root tomato plant (trial B). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

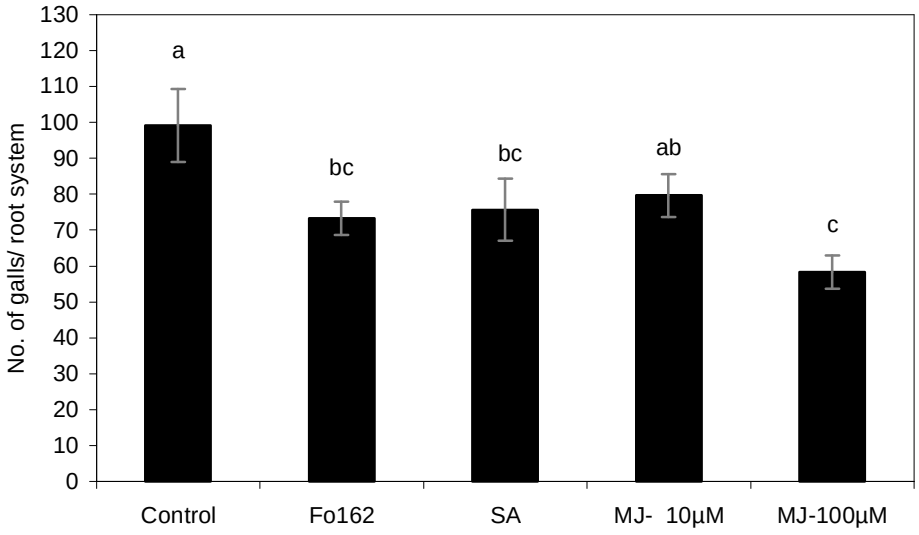

Figure.8. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on nematode galls / root system at the responded side of a split-root tomato plant (trial C). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

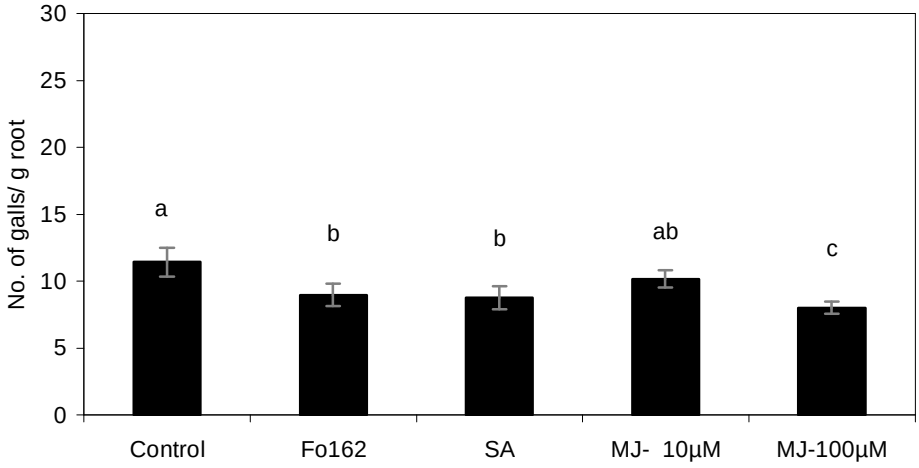

Figure.9. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on nematode galls / g root at the responded side of a split-root tomato plant (trial C). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

The influence of the biological and the chemical elicitors on reproduction of *M. incognita* was determined in trial C, by counting the number of egg masses found on the root systems. The results showed that Fo162, 200 µM SA and 100µM MJ caused significant reductions in the number of egg masses per root system (Fig.10) as well as in the number of egg masses per g root (Fig.11). The highest reduction was observed in the treatment with 100 µM MJ followed by Fo162 and then 200 µM SA. The application of 10 µM MJ did not affect the production of the egg masses when compared to the control.

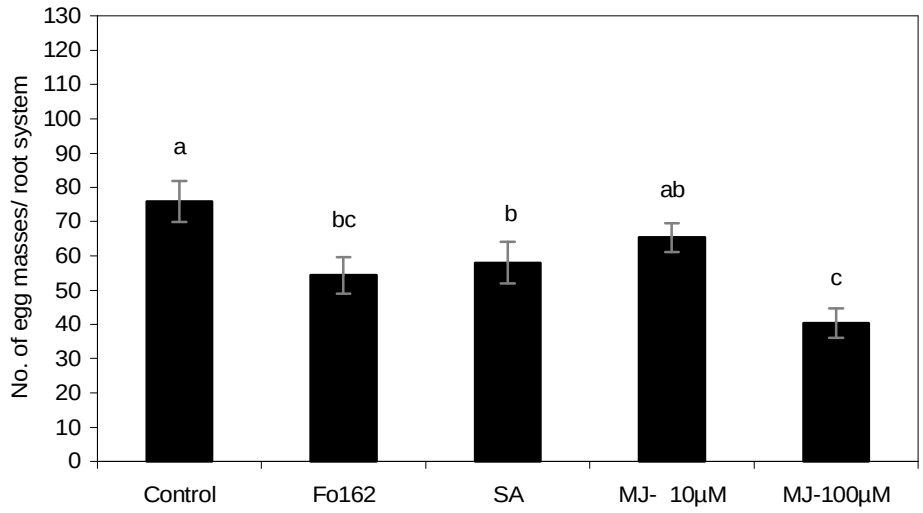

Figure.10. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on egg masses / root system at the responded side of a split-root tomato plant (trial C). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

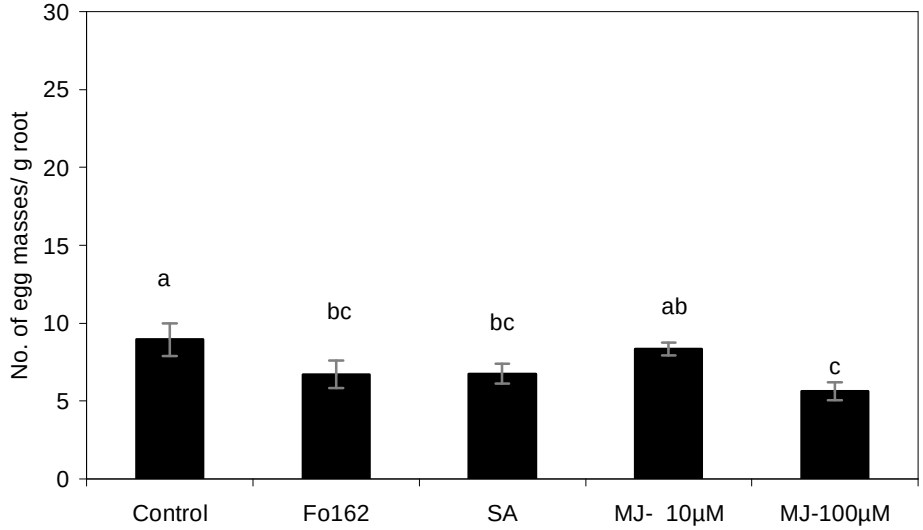

Figure.11. Effects of *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors on egg masses / g root at the responded side of a split-root tomato plant (trial C). Means with different letters are significantly different based on Tukey test (*p* 0.05; n= 6).

6.3.4. Plants transpiration

Tomato temperatures, reflecting the transpiration levels and water stress, were evaluated on plants of trial C, 14, 21 and 28 days post nematode inoculation. After 14 days the application of 200 µM SA and Fo162 caused significant reductions in leaf temperature compared to the control plants, (Fig.12.A). Conversely treatment with 100µM MJ caused significant increases in the plant temperature compared to the control. The tomato plants treated with 10µM MJ showed no temperature changes when compared to the control.

21 days after nematode inoculation, plant temperature in the 100 µM MJ treatment increased significantly compared to the control while Fo162 decreased the plants temperatures. 200 µM SA and 10 µM MJ had no significant effect on plants temperatures when compared to the control, (Fig.12.B).

28 days post nematode inoculation, 100 µM MJ caused significant increases in plant temperature whereas 200 µM SA, Fo162 and 10µM MJ reduced plant temperatures when compared to the control, (Fig.12.C).

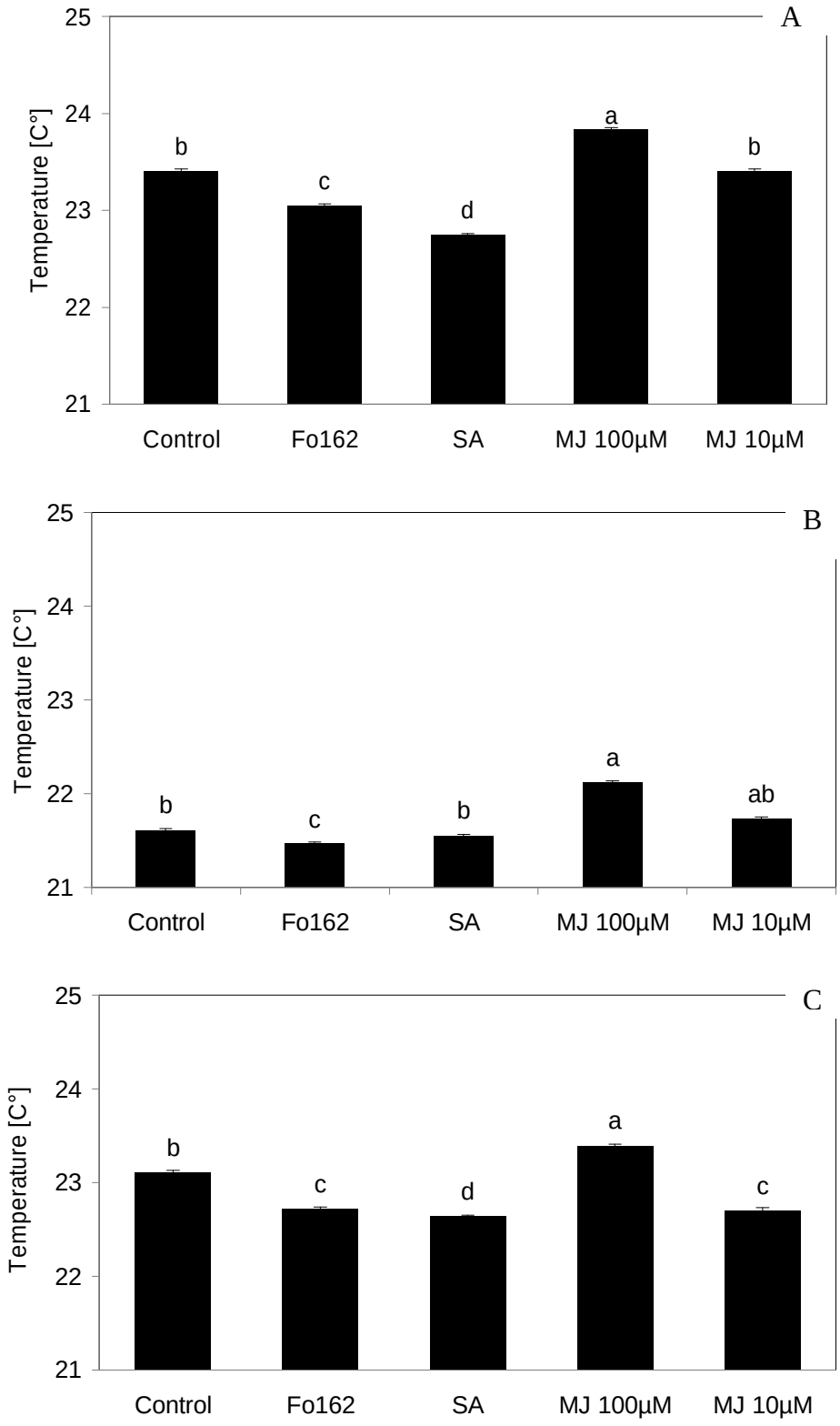

Figure.12.Tomato temperatures recorded 14 days (A), 21 days (B) and 28 days (C) after *Meloidogyne incognita* inoculation in plants treated with *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors. Means with different letters are significantly different based on Tukey test (*p* 0.05; n = 6).

6.3.5. Chlorophyll content

The intensity of leaf colour, indicative of chlorophyll synthesis, was measured in the plants of trial C, 14, 21 and 28 days after nematode inoculation. At 14 days post nematode inoculation, the highest chlorophyll content of in leaves was observed in the Fo162 inoculated plants compared to the control. In the 100µM MJ treated plants a lower level of chlorophyll was detected compared to the control. No significant differences with respect to leaf chlorophyll content were recorded in the presence of either 200 µM SA or 10µM MJ, (Fig.13.A).

Lower levels of chlorophyll were detected 21 days after nematode inoculation in tomato treated with 100µM MJ compared to the untreated control. The application of SA significantly increased the level of green content in the tomato leaves, while both Fo162 and 10 mM MJ had no significant effects, (Fig.13.B).

Similar results were recorded again when chlorophyll content was determined 28 days after nematode inoculation, (Fig.13.C). At this measurement time, 100 µM MJ reduced the chlorophyll content in the leaves, while 200 µm SA increased it compared to the control. No significant differences were recorded for Fo162 and 10µM MJ.

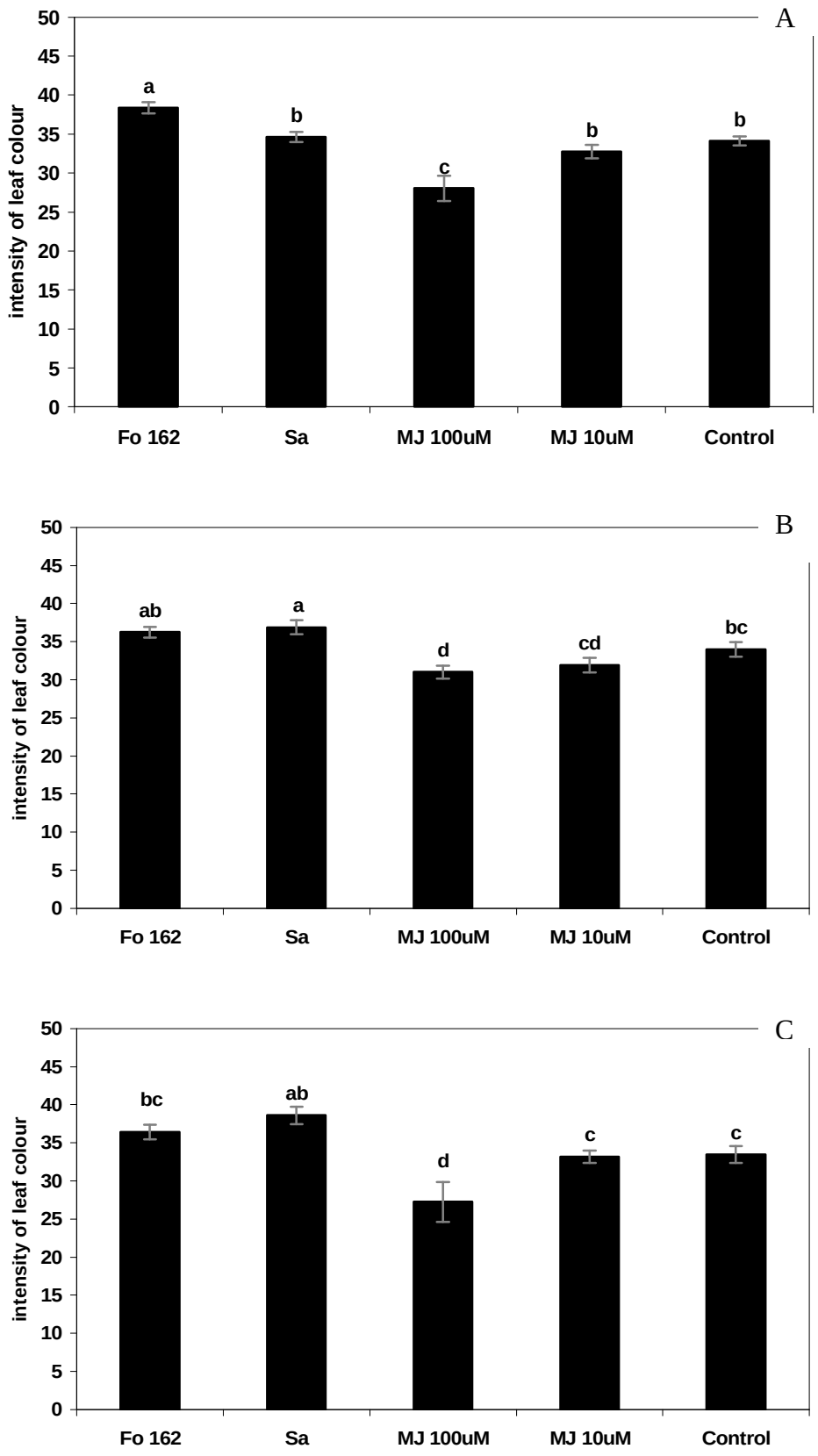

Figure.13. Intensity of leaf colour recorded 14 days (A), 21 days (B) and 28 days (C) after *Meloidogyne incognita* inoculation in plants treated with *Fusarium oxysporum* (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) elicitors. Means with different letters are significantly different based on Tukey test (*p* 0.05; n = 6).

6.4. Discussion

6.4.1. Root growth and nematode infection

In this study the ability of the mutualistic endophyte *Fusarium oxysporum* 162 (Fo162), salicylic acid (SA) and methyl jasmonate (MJ) to induce resistance in tomato against *M. incognita* has been tested using a split-root experiment. The influence of the application of Fo162 , the SA and MJ on root growth at the inducer and responder side of the split-root tomato plant also was determined.

The results showed that the biological inducer, Fo162, did not affect root growth at the inducer side while the chemical inducers, MJ and SA, significantly inhibited the root growth when compared to the control (6.3.1).

At the responder side, no significant differences in root weight were detected between the control and the Fo162 or SA treated plants. Only the application of 100 µM MJ significantly reduced root growth at the responder sides in two of the three trials, (6.3.2).

The biotic Fo162 and abiotic elicitors SA and MJ led to significant reductions in nematode infection. The highest reduction in both nematode galls and egg masses number was observed on plants treated with 100µM MJ. In trial A, the reduction in number of galls/ root system at the responder side reached approximately 67, 75, and 91% in the presence of Fo162, SA and MJ, respectively. In the other two trials, the effect of the biotic and abiotic elicitors on the nematode infection was less pronounced. However, these trials have been conducted at different times in the green house where the temperatures and humidity varied with over time. This could explain some of the variation which was observed between the conducted trials.

Overall, plant treatment with both the biological inducer, Fo162, and the chemical inducers, SA and MJ, caused significant reductions of *M. incognita* infection on tomato plants.

These results are similar to those obtained by Dababat and Sikora (2007) who found that inoculation of tomato plants with the non-pathogenic fungal endophyte *F. oxysporum* strain 162 resulted in a significant reduction of nematode infection. They hypothesized that this was in part due to induced resistance in the first 2-3 weeks after fungal inoculation.

Molinari and Loffredo (2006) reported that the ability of exogenously applied SA to induce resistance to root-knot nematodes in tomato is controversial and it seems to be linked to the means of application. Malamy and Klessig (1992) as well as Ryals *et al.,* (1996) stated that SA

is an essential factor for establishment of SAR in plants. In 1995, Silverman *et al.,* reported that with the rice cultivars, the resistance levels of plants exhibiting constitutive expression of SA is positively correlated with SA levels. Similar results were recorded by Coquoz *et al.,* (1995) within potato plants.

Moreover, Cooper *et al.,* (2005) showed that jasmonic acid application induced the systemic defence response which led to reducing the avirulent nematode reproduction on susceptible tomato plants. Stawswick *at al.,* (1998); Vijayan *et al.,* (1998) and Thaler *et al.,* (2004) mentioned that jasmonates play a crucial rule in plant defenses against plant parasitic nematodes that invade root systems. Furthermore, Soriano *et al.,* (2004a,b) reported that methyl jasmonate reduced the susceptibility of spinach and oats plants to different plant parasitic nematodes. Van Wees *et al.,* (2000) mentioned that disease suppression against different pathogens was enhanced when ISR and SAR were activated simultaneously using chemical elicitors.

Therefore, according to the results obtained in the present studies, both ISR and SAR can play a role in controlling nematode infection on tomato plants when they are induced biologically or chemically.

6.4.2. Plants transpiration

Measuring leaf temperatures which is correlated with plant transpiration and water stress was carried out in trial C on elicitor treated and non-treaded plants. The thermal images of treated and untreated tomato plants which were examined 14, 21 and 28 days after *M. incognita* inoculation showed that the application of 100µM MJ caused significant increases in leaf temperatures when compared to the control. Conversely, the biological inducer, Fo162, and the chemical elicitor, SA, generally caused reductions in temperature when compared to the control. These results could be due to the influence of the elicitors on root growth of the treated tomato plants. Severe inhibition in root growth was observed on the plants treated with 100µM MJ, which might explain the reduced water uptake and water content.

6.4.3. Chlorophyll content

The results of the present study also showed that Fo162 and SA increased the chlorophyll content in the leaves of treated plants compared to the control. Conversely, 100µM MJ significantly reduced the leaf chlorophyll content.

Similar results were obtained in the study of Oka *et al.,* (1999). They found that soil drenching with AABA, BABA and GABA did not have any phytotoxic effect on foliage of tomato plant while the younger leaves of plants sprayed with jasmonic acid or methyl jasmonate were slightly chlorotic. They reported also that JA and MJ soil drenching caused necrotic symptoms on roots tips and slight chlorosis on leaves whereas SA did not show any phytotoxic symptoms.

The combined results of root growth (see results 6.3.1 and 6.3.2), leaf temperatures (see results 6.3.4) and chlorophyll content (see results 6.3.5) could explain why the highest reduction in nematode infection (see results 6.3.3) was observed in 100mM MJ treated plants. It is well known that plant parasitic nematodes are obligate parasites and therefore they are affected by the factors that affect the host plant vitality. Therefore, the negative effects of 100µM MJ on the overall vitality as well as on root growth of the treated plants together with the additive effects of ISR could be responsible for the high levels of reduction in nematode infection observed on the elicitor treated plants.

6.5. Conclusion

Based on the results of the present investigation the following can be concluded:

1- Different systemic resistance signaling pathways can be stimulated biologically by using the biocontrol agent Fo162 or chemically via the chemical elicitors SA and MJ in tomato plants that are responsible for reductions in root-knot nematode infection.
2- Both of ISR and SAR signaling pathways are functional separately in controlling *Meloidogyne incognita* infection on tomato plants.
3- Inducing ISR in tomato using the methyl jasmonate elicitor has a negative impact on root growth and physiological status of treated plants even when low concentrations were applied.

6.6. References

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7. Alterations in gene expression in tomato by using biotic and abiotic elicitors of systemic resistance against root knot nematodes

7.1. Introduction

In plants, currently two distinct mechanisms of systemic induced resistance are recognized, systemic acquired resistance (SAR) and induced systemic resistance (ISR). In general, SAR results in the accumulation of transcripts coding for pathogenesis related (PR) proteins, of which especially PR-1 (protein with unknown function), PR-2 (β-1,3-glucanase) and PR-5 (thaumatin-like protein) are considered key markers (Pieterse *et al.,* 2002; Vallad and Goodman, 2004) and is generally effective against biotrophic pathogens. ISR results in the synthesis of secondary metabolites, including proteinase inhibitors and antifungal compounds known as phytoalexins (Farmer and Ryan, 1992; Tamogami and Kodama, 2000) and the accumulation of transcripts coding for a specific subset of PR-proteins, like PR-4 (chitinase) and PR-12 (defensin) and is generally effective against necrotrophic pathogens and herbivorous insects. The accumulation of these transcripts and metabolites are therefore important markers for the type of induced resistance. SAR can be triggered by salicylic acid whereas ISR can be induced by jasmonic acid (JA), methyl jasmonate (MJ) and ethylene (Sticher *et al.,* 1997).

Although both responses follow different pathways and generally exclude each other, there is considerable cross-communication between these two defense responses, allowing the plant to fine tune its response, depending on the attacker (Pieterse and Van Loon, 2004). Additionally, SAR and ISR can be activated simultaneously in a host plant using specific elicitors (Glazebrook *et al.,* 2003; van Wees *et al.,* 1999 and 2000; Li *et al.,* 2003; Thomma *et al.,* 1998; and Ton *et al.,* 2002).

In the previous chapter we showed a systemic reduction in *M. incognita* colonization and egg mass production, when exposing tomato roots to methyl jasmonate (MJ) or salicylic acid (SA). Apparently, both ISR and SAR can play an important role in the defense against root knot nematode.

The objective of the current study was to compare the changes in gene expression taking place in tomato plants, in the presence of these chemical resistance elicitors and the biological resistance elicitor, *Fusarium oxysporum* strain Fo162, by genome array analysis.

7.2. Materials and Methods

7.2.1. Experimental design

Seeds of the tomato cultivar ´´Hellfrucht / Frühstamm``, which is susceptible to *M. incognita*, were sown separately in plastic pots containing autoclaved soil:sand mixture (1:2 v/v). Three weeks after sowing (when seedlings were a bout 25-30 cm in height), the shoots and the root systems were longitudinally split into 2 halves and each half was placed in a separate pot of the twin pots chamber (as described in chapter 6). One week after splitting the shoots and the roots, One side of the twin pots was subsequently inoculated with either the biological or with the chemical inducer. This pot was termed the inducer side, while the other pot was termed as the responder side. The inducer sides of 5 plants were then individually inoculated with 10^6 cfu/g soil of Fo162 (as described in chapter 2), or treated with 200 µM SA, 100 µM MJ or with tap water (control).

The plants treated with the chemical inducers at the inducer side were watered daily with the respective inducing solutions, while the control plants and the Fo162 inoculated plants were watered with tap water daily. At the same time, the responder sides were in all cases watered with only tap water.

Stock solutions of salicylic acid and methyl jasmonate substrates were prepared with concentrations of 20 mM and 10 mM respectively. These stocks were dissolved in water by shaking for 1 hour at the magnetic stirrer. These stock solutions were stored in the dark and used for preparing the desired concentrations immediately prior to watering. All chemicals were purchased from Sigma-Aldrich. Tomato plants were grown under greenhouse conditions at 25 ± 5 °C with 16 h diurnal light and once a week fertilized with 2g/L NPK (14:10:14).

The experiment was terminated three weeks after starting applying the biological and the chemical inducers. At the harvesting time, shoots and leaves of each tomato plant were collected, individually transferred into 15 ml plastic tubes and immediately frozen in liquid nitrogen. The roots of the inducer and responder sides were removed individually from the soil, washed gently with tap water for few seconds to release soil particles and also transferred into 15 ml plastic tubes and immediately frozen in liquid nitrogen. Plant materials (leaves and roots of responder sides) were lypholized using a freeze drier (Leybold Heraeus, Lyovac GT2) for 24 hours. The Freeze dried samples were stored at -80°C until RNA extraction.

7.2.2. RNA extraction

Total RNA was extracted from roots of the responder sides and leaves of three independent plants for each treatment. The selected plant material was ground in a 15 ml plastic tube using a metal spatula and vortex. Total RNA was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel, Düren Germany) and further purified using the NucleoSpin RNA Clean-up XS kit (Macherey-Nagel). This total RNA was stored at -80°C until further analysis.

7.2.3. Gene chip array hybridization and analysis

The concentration and quality control of the total RNA by the Nanodrop ND-1000 Spectrophotometer and Agilent 2100 Bioanalyzer, respectively, together with the hybridization to the genome array chip was performed by ServiceXS, Leiden, The Netherlands. The array used was the Affymetrix (Santa Clara, Ca, U.S.A) GeneChip Tomato Genome Array, containing 10,038 tomato probe sets to monitor the expression of 9,254 *Lycopersicon esculentum* genes plus 11 control genes. The RNA was labelled using the Affymetrix 3'IVT Express Kit and hybridized with the gene chip according to the manufacturer's recommendations. The Affymetrix Command Console (v1.1) and Expression Console software (v1.1) were used to measure the performance of the washing, staining and scanning of the chips. With Command Console Viewer the arrays were checked for the placing of the grid. For the QC of the labelling, the labelling controls (added to the RNA before labelling) were evaluated. For the QC of the hybridization, hybridization controls (added to the hybridization cocktail) were evaluated. All the data are within QC specs, according Affymetrix Command Console specifications.

The raw data files were further analyzed in Bioconductor (http://www.bioconductor.org/) using MADMAX (https://madmax.bioinformatics.nl) Significance analysis of microarrays (SAM) was used as a statistical technique (Tusher, Tibshirani and Chu, 2001) to determine whether changes in gene expression were statistically significant. For each treatment-control comparison, the genes with a false discovery rate (FDR) of $p < 0.05$ were declared significant. The resulting list containing the differentially expressed genes were further annotated using the NCBI UniGene database (http://www.ncbi.nlm.nih.gov/unigene)

7.3. Results

7.3.1. Gene expression associated with inducing systemic resistance in tomato plants using biotic and abiotic elicitors

Tomato plants treated continuously with salicylic acid (SA) or methyl jasmonate (MJ) or inoculated with the endophyte, *Fusarium oxysporum* Fo162 at the inducer side were analyzed with respect to the changes in gene expression using genome array analysis. Therefore both the roots at the responder side and the leaves were isolated, the total RNA isolated and further analyzed by hybridizing to the Affymetrix Tomato Genome Array Gene Chip. Quality control showed that all the data were within the specifications as defined by the according Affymetrix Command Console and the three biological replicates were statistically very similar. The expression of each significant gene for each treatment, SA, MJ and Fo162, was compared to that in the water control. This resulted in a list representing the statistically change in gene expression.

Obtained results from the array analysis showed that the biological inducer, Fo162, and the chemical elicitors, SA and MJ, all affected the expression level of a substantial number of genes, both in roots and leaves. In tomato roots, 323 genes were down regulated while 419 genes were up regulated within Fo162 inoculated plants when compared to the control, (Table.1). SA repressed the expression of 194 genes in the roots while it increased the expression level of other 159 genes. With MJ, 268 genes were down regulated while 369 genes were up regulated compared to the control.

In the leaves, the highest number of re-regulated genes (2697 genes) was observed for the plants inoculated with Fo162. MJ and SA altered the expression levels of 2590 and 830 genes, respectively, (Table.1).

Table.1. Total number of up- and down- regulated genes in the leaves and the roots of tomato plants treated with Fo162, SA and MJ

	roots		leaves	
Treatemnet	down-regulated	up-regulated	down-regulated	up-regulated
Fo162	323	419	1626	1371
SA	194	159	469	361
MJ	268	369	1534	1056

When the differentially regulated genes in all three treatments were compared for the roots, it was found that that the expression level of 33 genes in the roots was down-regulated (Table.2) and the expression of another 33 genes were up-regulated in the roots of treated tomato plants with the biological inducer, Fo162, or both chemical inducers, SA and MJ, (Table. 3). Remarkable, when the expression was altered in all three treatments,

they were almost all altered in the same direction, except for Les.12053, Les.4345, Les.5506 and Les.1493.

Table.2. Fold changes (log.2 values) of down-regulated genes in the roots of treated tomato plants with *Fusarium oxysporum* 162, salicylic acid and methyl jasmonate, compared to the water control.

Gene accession	Fold changes			Gene function
	Fo162	SA	MJ	
Les.5567	-15,2	-6,5	-33,2	phosphoglycerate/bisphosphoglycerate mutase family protein
Les. 23605	-14,2	-4,9	-25,0	acyl-activating enzyme 11 (AAE11)
Les.5884	-11,2	-7,2	-6,5	na
Les.8632	-10,1	-7,3	-10,1	GCN5-related N-acetyltransferase (GNAT) family protein
Les.11125	-8,7	-7,1	-8,9	na
Les.14142	-8,2	-7,3	-6,4	na
LesAffx.37595	-7,8	-4,7	-5,0	na
Les.12053	1,0	-5,0	10,8	ATMGL; catalytic/ methionine gamma-lyase
Les.5608	-5,9	-11,1	-6,1	bile acid:Na+ symporter family protein
Les.5608	-5,5	-11,5	-4,5	na
Les.11107	-5,4	-4,8	-3,2	na
LesAffx.21383	-5,3	-6,0	-6,1	na
Les.1609	-5,2	-5,9	-5,1	glycosyl hydrolase family 17 protein
Les.11107	-4,1	-3,6	-3,4	na
Les. 13397	-3,9	-6,0	-10,3	ELF4 (EARLY FLOWERING 4)
Les.4779	-3,8	-2,4	-2,5	mitochondrial substrate carrier family protein
Les.101	-3,7	-3,5	-2,3	GTP cyclohydrolase II / 3,4-dihydroxy-2-butanone-4-phosphate synthase (ribA)
Les.11171	-3,5	-4,5	-2,8	UDP-glucoronosyl/UDP-glucosyl transferase family protein
Les.4807	-3,5	-3,3	-2,8	GRAM domain-containing protein / ABA-responsive protein-related
Les.1609	-3,0	-2,5	-2,5	glycosyl hydrolase family 17 protein-
Les.4345	-2,9	3,7	-6,8	CAB1 (CHLOROPHYLL A/B BINDING PROTEIN 1); chlorophyll binding-light-harvesting complex II chlorophyll a-b binding protein M3
Les.8849	-2,9	-2,7	-3,3	cytochrome f
Les.12389	-2,8	-3,6	-3,6	glycosyl transferase family 17 protein
Les.450	-2,7	-3,6	-2,6	ICK6/KRP3 (KIP-RELATED PROTEIN 3); cyclin binding / cyclin-dependent protein kinase inhibitor
Les.13807	-2,6	-3,2	-1,8	predicted protein-heat shock transcription factor 1
Les.2667	-2,6	-3,1	-2,6	WRKY33 (WRKY DNA-binding protein 33); transcription factor
Les.3670	-2,5	-2,2	-2,0	GPA1 (G PROTEIN ALPHA SUBUNIT 1); signal transducer
Les.13504	-2,4	-2,6	-1,9	leucine-rich repeat family protein
Les.5506	-2,3	3,6	4,3	O-diphenol-O-methyl transferase, putative
Les.3415	-2,1	-2,3	-1,9	PDS1 (PHYTOENE DESATURATION 1); 4-hydroxyphenylpyruvate dioxygenase
Les.5168	-2,1	-2,2	-1,9	cupin family protein
Les.6080	-1,8	-2,0	-1,8	phosphatidic acid phosphatase-related / PAP2-related
Les.5485	-1,6	-1,7	-2,2	TET8 (TETRASPANIN8)

na= not annotated.

Table.3. Fold changes (log.2 values) of up-regulated genes in the roots of treated tomato plants with *Fusarium oxysporum* 162, salicylic acid and methyl jasmonate compared to the water control.

Gene accession	Fold changes			Gene function
	Fo162	SA	MJ	
Les.1493	1,4	1,4	-1,8	NIT4 (NITRILASE 4); 3-cyanoalanine hydratase/ cyanoalanine nitrilase / indole-3-acetonitrile nitrilase/ nitrilase/ nitrile hydratase
Les.2791	1,7	1,9	1,7	ATABC1 (ATP BINDING CASSETTE PROTEIN 1); ATPase, coupled to transmembrane movement of substances / protein binding / transporter
Les.3195	1,8	1,5	1,7	SEX1 (STARCH EXCESS 1); alpha-glucan, water dikinase
Les.2331	1,8	2,2	4,2	oxidoreductase family protein
Les.5954	1,9	1,9	2,4	MKK9 (MAP KINASE KINASE 9); MAP kinase kinase/ kinase/ protein kinase activator
Les.5017	2,1	1,8	2,1	MYB59 (MYB DOMAIN PROTEIN 59); DNA binding / transcription factor
Les.9785	2,1	2,3	2,0	transferase, transferring glycosyl groups
Les.1258	2,2	2,2	4,5	POP2 (POLLEN-PISTIL INCOMPATIBILITY 2); 4-aminobutyrate transaminase/ 4-aminobutyrate:pyruvate transaminase
Les.1163	2,2	1,8	2,3	transcription factor
Les.2650	2,3	2,0	1,7	unknown protein
Les.1205	2,3	4,3	3,1	PREDICTED: hypothetical protein
Les.19379	2,3	2,9	2,6	predicted protein
Les.1205	2,4	3,9	3,0	repeated
Les.631	2,5	4,2	2,2	amino acid transporter family protein
Les.15182	2,6	1,8	1,8	unknown protein
Les.631	2,6	5,0	2,7	repeated
Les.2316	2,6	4,1	6,3	PREDICTED: hypothetical protein
Les.1650	2,8	3,5	16,9	tropinone reductase, putative / tropine dehydrogenase, putative
Les.4085	3,0	2,5	2,9	LHW (LONESOME HIGHWAY); protein homodimerization/ transcription activator/ transcription factor
Les.16464	3,1	3,3	19,5	Transcribed locus, weakly similar to XP_002315876.1 predicted protein and NP_177795 OPR2 (12-oxophytodienoate reductase 2); 12-oxophytodienoate
Les.22	3,1	2,9	2,0	12-oxophytodienoate reductase (opr)
Les.9144	4,3	12,2	110,6	In2-1 protein, putative
Les.5999	4,8	3,7	3,3	zinc finger (B-box type) family protein
Les.2316	4,9	7,1	14,8	repeated
Les.1122	5,1	5,4	6,8	_
Les.23330	5,5	4,1	4,2	sarcosine oxidase family protein
Les.1314	7,5	21,7	9,9	EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1); DNA binding / transcription factor
Les.8161	8,0	12,3	5,5	LBD41 (LOB DOMAIN-CONTAINING PROTEIN 41)
Les.6954	12,5	7,3	16,4	DNA-binding family protein
Les.22696	17,2	6,2	6,1	hydroxyproline-rich glycoprotein family protein
Les.3388	17,5	7,0	33,7	CYP94C1; fatty acid (omega-1)-hydroxylase/ oxygen binding
Les.4923	31,8	71,7	37,0	LHY (LATE ELONGATED HYPOCOTYL); DNA binding / transcription factor.- cca1b circadian clock protein CCA1b
Les.207	2,1	2,4	1,4	CYP76C5; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding

In the leaves of Fo162 inoculated plants, the fold changes in the gene expression compared to the control ranged from -130 to 45 with the genes of Les.2792 and Les.19563, respectively. However, the putative function of Les. 2792 was not assigned while Les.19562 was found to be a member of GCN5-related N-acetyltransferase (GNAT) family protein and therefore was considered a transcriptional co-activator. With SA treated plants, the fold changes in the gene expression compared to the control ranged from -57 to 119. This was observed for Les.5608 and Les.4057, respectively. The Les.5608 was found to be a member of bile acid: Na+ symporter family protein, which is involved in ion transport. The Les.4057 gene was annotated as *Lycopersicon esculentum* DB29, but no further information could be found. In MJ treated plants, the fold changes in the gene expression compared to the control ranged from -130 to 2081 with Les.2792 and Les.840, respectively. Until now, both genes were not annotated.

When the differentially regulated genes in all three treatments were compared for the leaves, it was found that the expression level of 422 genes was altered, of which 146 genes were up-regulated, (Fig.1), and 276 genes were down-regulated, (Fig.2). The highest number of up regulated genes, 27, was observed for the genes related to developmental pathways followed by those involved in the signalling pathways (24 genes). When the down regulated genes were further analyzed, this showed that the highest number of genes, 60, was related to protein synthesis.

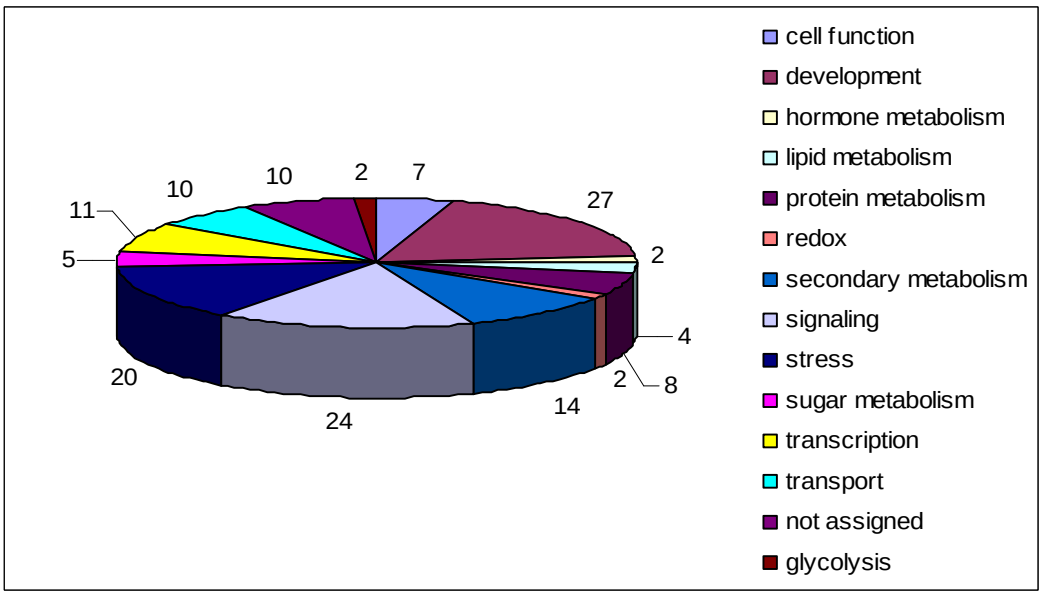

Figure.1. up regulated genes in the leaves of treated plants with Fo162, SA and MJ.

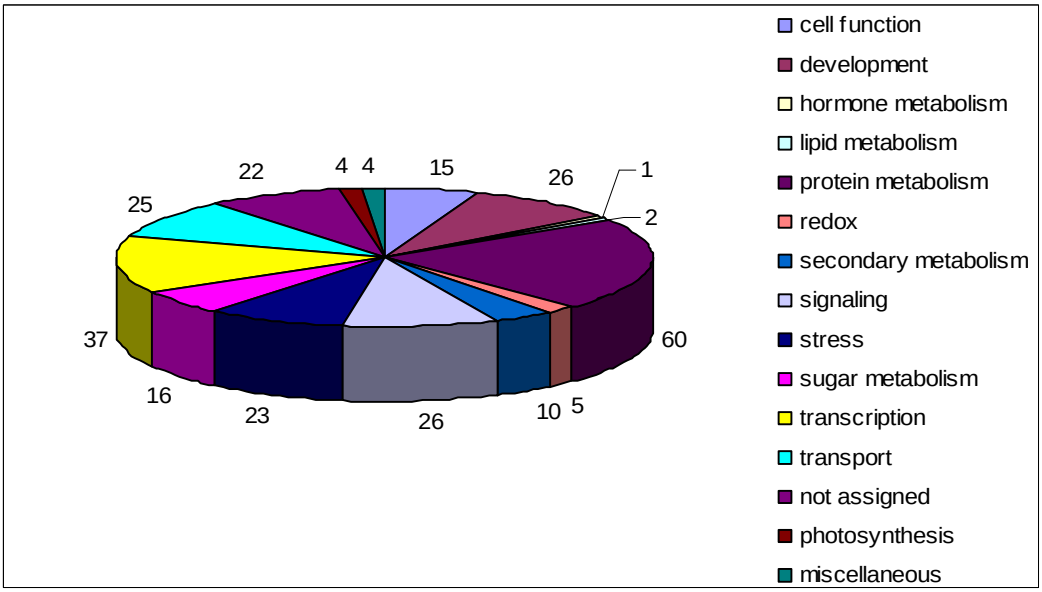

Figure.2. Down regulated genes in the leaves of treated plants with Fo162, SA and MJ.

7.3.2. Gene expression associated with systemic defences pathways in the elicitor treated plants

When the genes, of which the expression level had altered in the roots and leaves of both Fo162 inoculated and SA or MJ treated plants, were further analyzed with respect to their putative functions, 9 different genes were found which could be related to resistance responses in tomato plants, (Table.4). In the roots, SA increased the transcript levels of 8 genes related to resistance while only one gene (Les.3710) was down regulated. In the leaves, Fo162 stimulated the expression of 5 genes related to defences while only one gene (Les.3710) was down regulated. MJ affected the transcript level of only one gene (Les.8831).

Table.4. Changes in expression of the genes related to tomato resistance within treated plants with Fo162, SA and MJ compared to the water control.

Gene accession	Fold changes in leaves			Fold changes in roots			putative function
	Fo162	SA	MJ	Fo162	SA	MJ	
Les.8831	2,1	2,5	1,9	nc	1,2	nc	ATMRP4 (Arabidopsis thaliana multidrug resistance-associated protein 4).
Les.3710	-5,9	nc	nc	nc	-3,0	nc	LAG1 (Longevity assurance gene 1)
Les.3709	1,5	nc	nc	nc	1,1	nc	LAG13 (LAG1 LONGEVITY ASSURANCE HOMOLOG 3)
Les.75	nc	nc	nc	nc	2,1	nc	Plant resistance protein (Mi-1.1)
Les.12390	nc	nc	nc	nc	1,2	nc	disease resistance-responsive family protein
Les.4039	2,6	nc	nc	nc	1,1	nc	Lycopersicon esculentum resistance complex protein I2C-4 (I2C-4) possible pseudo mRNA sequence
Les.20018	1,5	nc	nc	nc	1,3	nc	ATMRP1 (Arabidopsis thaliana multidrug resistance-associated protein 1);
Les.3990	2,1	nc	nc	nc	2,3	nc	Hero resistance protein 1 homologue (help1)
Les.3989	nc	nc	nc	nc	2,3	nc	Lycopersicon esculentum partial mRNA for hero resistance protein 2 homologue (help2 gene)

nc= the expression level of the gene was not significantly changed compared to the control.

7.3.3. Gene expression associated with chlorophyll synthesis

In the roots, 2 genes related to chlorophyll synthesis were down-regulated in the presence of MJ, while only one gene was down-regulated with Fo162 inoculated plants. Conversely, SA increased the expression level of 12 genes which were related to chlorophyll synthesis. In the leaves, Fo162 increased the transcript levels of 5 related genes to chlorophyll, with a fold change of up to 6.4 with Les.1603. SA increased the transcript levels of two chlorophyll genes, with a fold change of up to 4.9 with Les.1603. With MJ, the fold changes in the gene expression of related genes to chlorophyll reached up to 2.7 with Les.19928, (Table.5).

Table.5. Changes in expression of the genes related to chlorophyll synthesis within treated tomato plants with *Fusarium oxysporum* 162, salicylic acid and methyl jasmonate compared to the control.

Gene accession	Fold changes in leaves			Fold changes in roots			putative function
	Fo162	SA	MJ	Fo162	SA	MJ	
Les. 6480	2,3	2,5	2,0	nc	1,6	nc	HCF101 (HIGH-CHLOROPHYLL-FLUORESCENCE 101); ATP binding
Les.19108	nc	nc	1,8	-2,0	1,9	nc	chlorophyll A-B binding protein CP29 (LHCB4) - light-harvesting complex II protein Lhcb4
Les.9969	2,3	nc	nf	nc	1,8	nc	LHCB2.2 (Photosystem II light harvesting complex gene 2.2); chlorophyll binding
Les.11941	2,0	nc	nc	nc	1,2	nc	DVR (PALE-GREEN AND CHLOROPHYLL B REDUCED 2);
Les.19928	4,7	nc	2,7	nc	2,2	-2,9	LHCB6 (LIGHT HARVESTING COMPLEX PSII); chlorophyll binding
Les.1603	6,4	4,9	nc	nc	1,4	nc	LHCA3 (Photosystem I light harvesting complex gene 3); chlorophyll binding
Les.3132	nc	nc	-1,8	nc	1,3	nc	PIFI (POST-ILLUMINATION CHLOROPHYLL FLUORESCENCE INCREASE)
Les.10701	nc	nc	nc	nc	1,3	nc	ATG4/CHLG/G4 (CHLOROPHYLL SYNTHASE); chlorophyll synthetase
Les.233	nc	nc	nc	nc	4,6	nc	LHCB2.2 (Photosystem II light harvesting complex gene 2.2); chlorophyll binding.
Les.19372	nc	nc	nc	nc	4,3	-5,6	Chlorophyll a/b binding protein precursor (LOC544310)
Les.19336	nc	nc	nc	nc	3,2	nc	LHCA3 (Photosystem I light harvesting complex gene 3); chlorophyll binding
Les.15660	nc	nc	nc	nc	1,3	nc	Red chlorophyll catabolite reductase (rccr).

nc= the expression level of the gene was not significantly changed compared to the control.

7.3.4. Gene expression associated with water stress

Influence of SA, MJ and Fo162 on the expression of some involved genes in the water stress responses of tomato plants was monitored. Results showed that only one gene (Les. 9808) was down regulated with SA while all other genes were up regulated. The transcription levels of these genes reached up to 4.6 with Les.13064 in treated plants with SA, (Table. 6).

Table.6. Changes in the expression of the genes related to water stress within treated tomato plants by *Fusarium oxysporum* 162, salicylic acid and methyl jasmonate compared to control.

Gene accession	Fold changes in leaves			Fold changes in roots			putative function
	Fo162	SA	MJ	Fo162	SA	MJ	
Les.3195	1,6	nc	nc	1,8	1,5	1,7	Glucan water dikinase (GWD)_* SEX1 (STARCH EXCESS 1)
Les.19468	nc	nc	nc	nc	1,9	nc	PIP2A (PLASMA MEMBRANE INTRINSIC PROTEIN 2A); water channel
Les.13064	3,6	4,6	4,2	nc	1,3	nc	TMP-C (PLASMA MEMBRANE INTRINSIC PROTEIN 1;4); water channel
Les.119	nc	nc	3,9	nc	1,5	nc	PIP1;5/PIP1D (plasma membrane intrinsic protein 1;5); water channel_* Aqp2 protein (Aqp2)
Les.314	nc	nc	nc	nc	2,0	nc	PIP3 (PLASMA MEMBRANE INTRINSIC PROTEIN 3); water channel.
Les.9808	nc	nc	nc	nc	-1,7	nc	BETA-TIP (BETA-TONOPLAST INTRINSIC PROTEIN); water channel.
Les.9936	nc	nc	nc	nc	1,5	nc	TIP4;1 (tonoplast intrinsic protein 4;1); water channel.

nc= the expression level of the gene was not significantly changed compared to the control.

7.4. Discussion

7.4.1. Gene expression associated with inducing systemic resistance in tomato plants using biotic and abiotic elicitors

In this genome array study, the effect of exogenous application of salicylic acid (as inducer of SAR pathway), methyl jasmonate (as inducer of ISR pathway) and the mutualistic endophyte *Fusarium oxysporum* strain 162 (as biological inducer of systemic resistance against *M. incognita*) on the expression of 9,254 *Lycopersicon esculentum* genes in leaves and roots was monitored in a split root experiment. This number of genes is by no means covering the complete gene content of tomato. Furthermore, not all probes found on the gene chip have been annotated with respect to gene function. Nevertheless, the analysis gives a good insight in the overall changes taking place in the tomato plant in the presence of the biotic and abiotic elicitors of systemic resistance against root knot nematodes.

When applied to the roots (inducer side), we observed that all three elicitors, Fo162, SA and MJ, altered the transcript level of a substantial number of genes in both the responder roots and leaves.

Fo162 showed an alteration in transcription level of more than 29% of the genes. This systemic effect is remarkable, since Fo162 behaves like an endophyte and, consequently, does not cause any substantial phenotypic changes in the plant. This percentage is even greater that the alterations caused by MJ (25%). As demonstrated in the previous chapter MJ leads to phenotypic chances in the tomato plant. In this light the effects caused by SA with 9% re-regulation of gene expression can be considered modest. The result with the chemical elicitors corroborate with other studies of the past years with microarray analysis of both compatible and incompatible plant-pathogen interaction showed that hundreds of genes were up- and down regulated (van Loon *et al.,* 2006). These studies showed that the exogenous supply with SA and methyl jasmonate resulted in changing the expression level of many different genes sets which some of them mediate different kinds of tomato systemic resistance. Nowadays, it is known that defense mechanism is regulated with complex and separate different pathways (Luo *et al.,* 2009).

These high percentages only applied to the tomato leaves. The number of genes showing a change in expression level in the roots was significantly lower for al three treatments and ranged from 3.5 for the SA treatment to 7.3 % for the Fo162 inoculation. Apparently, the systemic responses to the inducers are more pronounced in leaves when compared to the roots.

Nevertheless, as demonstrated in the previous chapter, systemic resistance against root knot nematode colonization did occur with all three elicitors. When the genes were selected that were altered in expression level in the roots for all three elicitors, we found only 66 genes in common, which had almost all altered in the same direction with the three elicitors. However, within this list we could not discover genes that could be directly associated with nematode resistance. There may be several reasons for that. First, the function of some of the genes is currently not known. Second, since the array does not cover all genes within the tomato genome, we may have missed the relevant genes. Third, the plant may only be primed and the relevant resistance genes will only become visible when the nematode is entering the root system. In this case, we would have had to include RNA from a split root experiment, infected with nematodes at the responder side. Williamson and Hussey (1996) reported that many of the genes involved in the interaction between root-knot nematode and their hosts. These genes were found to be members of gene families and have a complex regulation patter. However, to avoid the influences in the gene expression during the interactions between *M. incognita* and tomato plants, the array analysis was conducted using elicitor treated plants in the absence of nematode colonization. Fourth, the genes playing a role in nematode resistance are expressed mainly in the leaves and not in the roots.

When the genes were selected that were in common and thus altered in expression level in the leaves for all three elicitors, we found 422 genes. Like in the roots, almost all these genes were altered in the same direction with the three elicitors. Their gene products had an array of functions, ranging from basic cell function and development to stress and protein metabolism.

In the leaves several interesting alterations in gene expression were found, that may support the suggestion that nematode defense related factors originate from the leaves. Two related genes to *Meloidogyne*-induced giant cell altered in their expression in the presence of the elicitor, Les.4082 and Les.4032. Les.4082 was always down-regulated while the other one, Les.4032, was always up-regulated with all treatments (data not shown). This variation within the expression of those two genes, which gene products can control or are involved in giant cell formation (Bird and Wilson, 1994) may affect the pericycle tissues. Parizot *et al.,* (2008) reported that the pericycle is a heterogeneous cell layer with two groups of cells set up in the root meristem by the same genetic pathway controlling the diarch organization of the vascular system. Although, additional information about the true function of these genes is necessary, which potentially leads to new insights

regarding giant cell formation and plant resistance, it indicates that genome array analysis can support to identify crucial genes playing a role in plant-pathogen interactions.

The transcription of defense-related genes was found to responsible for the accumulation of different defense compounds and antimicrobial activities with host plant (Hammond-Kosack and Jones, 1996). It was found also that the exogenous application of salicylic acid as well as jasmonates resulted in activation of different defense-related genes. For example, Uknes *et al.,* (1992) reported that SA affecting the gene expression of pathogenesis-related proteins of the families PR-2, PR-5 and PR-1 while other studies (Penninckx *et al.,* 1996; Thomma *et al.,* 1998; and Melan *et. al.,* 1993) showed that jasmonate induces the expression of other different gene sets in *Arabidopsis* plants i.e. *Hel*, *ChiB* and *Pdf1.2* as well as *Lox1* and *Lox2* which are related to defenses of host plants.

Although some of the PR-genes e.g. 1b1, p23, 5x, 1a1, P2 and P4 were indeed present on the tomato array. Significant changes in the expression of these genes were observed only in the root of SA treated plants while no significant changes were detected with the other elicitors. This may also be due to the prolonged exposure to the elicitors. Normally the up-regulation in expression of these genes are observed shortly after the application of the elicitors.

Our results also agree with those of with van Wees *et al.,* (2000), who demonstrated that different defence pathways can be activated within plants treated with distinct inducing agents. Their results illustrated also that both of SAR and ISR systemic resistance which are mediated by SA and MJ signalling pathways, respectively provided an attractive tool for the improvement of disease control. Moreover, they added that the SAR- and ISR-regulatory gene *Npr1* was over expressed (and/or activated) when both of ISR and SAR signalling pathways were stimulated during *Arabidopsis* infection with non-pathogenic rhizobacteria and pathogen infection, respectively. In advanced research, Branch *et al.,* (2004) investigated the role of SA, which plays a crucial role in alteration of many of *R* gene-mediated resistance responses in tomato and they demonstrated that SA was required for *Mi*-mediated nematode resistance in tomato roots. SA, JA and ET are three hormones which accumulate in response to pathogen infection or herbivore damage, leading to the activation of distinct and partly overlapping sets of defense-related genes. The studies conducted with *Arabidopsis* wild type plants and the SA-, JA-, or ET- signalling mutants *Arabidopsis* plants that were infected by *Pseudomonas syringae* pv. *maculicola*

showed an evidence for the substantial cross-talk between the signalling pathways which were mediated by the three hormones (Glazebrook *et al.,* 2003).

Moreover, van Wees *et al.,* (1999) reported that inducing SAR pathways in *Arabidopsis* plants, by pre-inoculated lower leaves with avirulant isolate of *Pseudomonas syringae* pv. tomato, affected the infection with pathogenic *Pseudomonas syringae* pv. tomato isolate in the upper leaves and also resulted in increasing the expression levels of different tested genes which some of them related to ISR pathway while the other are related to SAR pathway.

7.4.2. Gene expression associated with chlorophyll synthesis and water stress

The regulation levels of genes related to some vital growth signs like chlorophyll accumulation and transpiration level in tomato plants were also analyzed to find out if there is a correlation between the induction to different types of tomato systemic resistance and some of the overall physiology of the plant.

As observed before with related genes to defense pathways, the highest number (12 genes) of up regulated related genes to chlorophyll synthesis was observed with SA treated plants followed by plants inoculated with Fo162 (5 genes). In contrast, MJ inhibited the regulation of three genes that were related to chlorophyll pathway. These findings corroborate with the results obtained from measuring the accumulation of chlorophyll pigment in the leaves of treated tomato plants with SA, MJ and Fo162 (see previous chapter).

The changes in the expression of genes related to water stress suggest that plants treated with SA a less exposed to water stress. This is in agreement with the thermal imaging data, which reflects the water content in tomato plants and thus indicates the transpiration levels (see previous chapter). Alfano *et al.,* (2007) used high-density oligonucleotide microarrays to investigate the expression pattern of 15925 genes in tomato leaves obtained from inoculated plants with biocontrol agent *Trichoderma hamatum* 382 prior their inoculation with the pathogen. Their results detected up-regulation of different genes associated with biotic or abiotic stress as well as RNA, DNA and protein metabolism was detected also.

7.5. Conclusion

Based on the finding in the present investigation the following can be concluded:

1- Micro array analysis was a useful tool for determining the molecular changes within treated tomato plants with different inducers responsible for activation different types of systemic resistance.

2- The chemical elicitors, SA and MJ, and the biological inducer, Fo162, alter the expression of many genes which can play a certain role in the diseases resistance.

3- The highest number of genes which were altered in expression levels was detected within the plants leaves, especially the plants inoculated with Fo162.

4- Inducing the defence related genes in tomato plants using the biological or the chemical inducers also can affect the expression patterns of genes whose products are associated with chlorophyll synthesis and water stress.

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