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Interrelationships between mutualistic endophytic microorganisms, the root-knot nematode *Meloidogyne incognita* and the sap-sucking insect *Aphis gossypii* on tomato, squash and Arabidopsis

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Dedicated to my family for their permanent strong support…

Interrelationships between mutualistic endophytic microorganisms, the root-knot nematode *Meloidogyne incognita* and the sap-sucking insect *Aphis gossypii* on tomato, squash and Arabidopsis

The effects of single and combined applications of three endophytic microorganisms on the biocontrol of the root-knot nematode *Meloidogyne incognita* and the sap-sucking insect *Aphis gossypii* in tomato, squash and Arabidopsis were investigated. The studied endophytes were the fungus *Fusarium oxysporum* strain Fo162 (Fo162), the mycorrhiza fungus *Glomus intraradices* strain 510 (AMF) and the bacterium *Rhizobium etli* strain G12 (G12). In addition, factors that can negatively affect the interrelationships between the endophytic microorganisms in relation to their biocontrol activity were studied. Greenhouse experiments were conducted to investigate the biological control of *M. incognita* in tomato by single or combined applications of Fo162, AMF and G12. The individual application of each of the biocontrol agents resulted in a significant reduction in the number of *M. incognita* that penetrated into tomato roots. However, concomitant enhancement with Fo162 together with AMF or with G12 did not improve the reduction of *M. incognita* penetration. Triple-split-root experiments showed that the simultaneous, but spatially separated inoculation of both Fo162 and G12, also did not lead to a significant increase in the reduction of *M. incognita* penetration, when compared to the individual inoculation. In these experiments a reduction in Fo162 colonization also was observed, demonstrating that the growth of the antagonistic fungus can be systemically inhibited by the bacterium. Other experiments demonstrated that Fo162 and G12 root colonization restrained *M. incognita* development when the two organisms are present in the same root system. Greenhouse experiments were performed to study the biocontrol of *A. gossypii* in squash by single or combined applications of Fo162, AMF or G12. A small level of biological control activity toward *A. gossypii* was observed when the insects were exposed to squash plants inoculated with AMF. Conversely, Fo162 and G12 when present alone reduced significantly the population development of *A. gossypii* on squash. However, there was no evidence of a synergistic interaction toward the aphid when Fo162 together with AMF or with G12 were inoculated simultaneously to squash. Split-root experiments showed that the simultaneous, but spatially separated inoculation of both Fo162 and G12 did not improve the biocontrol of *A. gossypii*. *In vivo* and *in vitro* experiments were conducted to investigate the interrelationships between Fo162 and G12. The combined application, onto the same root system, of both Fo162 and G12 showed a significant reduction in tomato root colonization by Fo162. Moreover, the simultaneous but spatially separated inoculation of both endophytes, in a split-root system, resulted again in a reduction in Fo162 colonization, indicating that the growth of the fungus can be systemically inhibited by the bacterium. Antibiosis tests indicated that there was a reduction in radial growth of Fo162, when challenged with G12. *In vivo* and *in vitro* trails were then conducted on Arabidopsis to study the interrelationship between Fo162 and *M. incognita*. Results indicated that Fo162 was able to effectively colonize the Arabidopsis root system. Fo162 treatment resulted in a significant reduction in the number of *M. incognita* that penetrated into Arabidopsis roots. Split-root Arabidopsis experiments also demonstrated that Fo162 was able to systemically reduce *M. incognita* number of galls. Moreover, the colonization of Arabidopsis roots by Fo162 caused distinct plant growth enhancement activity. The results obtained demonstrated that the combination of "Arabidopsis-Fo162-*M. incognita*" could be used as a model system to elucidate the molecular basis of the interactions of these three organisms.

Interaktion zwischen mutualistischen endophytischen Mikroorganismen, der Wurzelgallennematode *Meloidogyne incognita* und dem Phloem-saugenden Insekt *Aphis gossypii* an Tomate, Zucchini und Arabidopsis

In der vorliegenden Arbeit wurden Einzel- und Kombinationsanwendungen endophytischer Mikroorganismen auf ihre biologische Kontrollaktivität gegenüber des Wurzelgallennematode *Meloidogyne incognita* und dem Phloem-saugenden Insekt *Aphis gossypii* an Tomate, Zucchini und Arabidopsis untersucht.

Die untersuchten Endophyten waren der Ascomycet *Fusarium oxysporum* Fo162 (Fo162), der Mycorrhizapilz *Glomus intraradices* 510 (AMF) und das Bakterium *Rhizobium etli* G12 (G12). Zusätzlich wurden Faktoren untersucht, die die Wechselbeziehung zwischen den genannten Endophyten und ihrer biologische Kontrollaktivität negativ beeinflussen können. Biologische Kontrolle von Einzel- und Kombinationsanwendung von Fo162, AMF und G12 gegenüber *M.incognita* an Tomatenpflanzen wurden in Gewaechshausexperimenten untersucht.

Einzel Anwendung der jeweiligen Endophyten, resultierte in einer signifikanten Verringerung des Eindringens von *M. incognita* in Tomatenwurzeln. Kombinierte Anwendung hatte jedoch keinen synergistischen Effekt auf die Biokontrolle gegenüber *M. incognita*. Auch eine räumliche Trennung der Endophyten in Triple Split-Wurzelexperimenten zeigten keinen synergistischen biokontroll Effekt gegenüber *M. incognita*. Jedoch, wird die Fo162 Kolonisierung an Tomatenwurzeln durch G12 systemisch signifikant reduziert.

Gewächshausexperimente wurden durchgeführt, um das biologische Verhalten von *A. gossypii* an Zucchini bei Einzel- und Kombinationsinokulation von Fo162, AMF, G12 zu untersuchen. Ein geringe biologische Kontrollaktivität konnte mit AMF gegen *A. gossypii* an Zucchini beobachtet werden. Fo162 und G12 hingegen konnten beide die Populationsentwicklung von *A. gossypii* signifikant reduzieren. Es gab jedoch keinen Nachweis für einen synergetischen Effekt durch eine kombinierte Applikation von Fo162 mit AMF oder G12. Split-Wurzel Experimente haben gezeigt, dass gleichzeitig aber räumlich voneinander getrennte Inokulation von Fo162 und G12 ebenfalls keinen synergetischen Effekt gegenüber *A. gossypii* hat.

Antibiosistest haben gezeigt, dass G12 das radiale Wachstum von Fo162 signifikant inhibiert. Die Ergebnisse demonstrieren, dass in Co-inoculations Strategien mit Fo162 und G12 auch die mikrobiellen Interaktionen berücksichtigt werden sollte. *In vivo* und *in vitro* Experimente wurden mit Fo162, Arabidopsis und *M.incognita* durchgeführt. Fo162 ist in der Lage das Wurzelsystem von Arabidopsis zu kolonisieren und resultierte in einer signifikanten Reduzierung von penetrierenden *M. incognita*. Split-Wurzel Experimente mit Arabidopsis zeigen, dass Fo12 die Anzahl an Gallen systemisch reduzieren kann. Darüber hinaus zeigten Fo162-kolonisierte Arabidopsis pflanzen einen characteristischen Phänotyp. Die Ergebnisse zeigen, dass Arabidopsis-Fo162-*M. incognita* als Modelsystem für Interaktionen zwischen der mutualistische Endophyt Fo162 und *M. incognita* genutzt werden könnte.

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Chapter 1
General introduction

1.1. Importance of tomato, squash and Arabidopsis

Tomato (*Lycopersicon esculentum* Mill) is one of the most important vegetable cultivated for human consumption. It is grown on every continent in the world in fields, greenhouses, plastic tunnels and in net houses (Wener, 2000). World production of tomato and its cultivated area has increased at a rate of 4.6 million metric tons year^{-1} and 41 thousand hectares year^{-1} in the period from 2000 to 2009 (Fig. 1.1). Thus, world production of tomato in 2009 exceeded 150 million metric tons and occupied approximately 4.4 million hectares (Fig. 1.1).

Increased production of tomato is related with important advances in production and processing technology. Additionally, modern breeding methods supported by molecular techniques are contributing importantly to shorten the development time for cultivars with plant resistance to pest and diseases. The increase in the importance of vegetables, including tomato, is evident in countries with rapidly expanding populations, e.g. Africa and Asia, where large amounts of land near urban centers are dedicated to vegetable production (Sikora and Fernandez, 2005).

Squash (*Cucurbita pepo* L.) is an annual herbaceous climbing plant, which belongs to the family Cucurbitaceae. This family includes many economically important vegetables such as cucurbits, cucumber, melon and watermelon; which are normally used as fruit, for cooking and for decoration (Jeffrey, 1990; Lira and Caballero, 2002; Wehner and Maynar, 2003). This group of vegetables is grown worldwide with worldwide production and cultivated area under pumpkins, squash and gourds in the period from 2000 to 2009 increasing by 9.64 thousand metric tons year^{-1} and 20.3 thousand hectares year^{-1}, respectively (Fig. 1.2). Therefore, world production of cucurbits type vegetables in 2009 exceeded 750 thousand metric tons and occupied approximately 1.7 million hectares (Fig. 1.2).

Arabidopsis thaliana is a small flowering plant native to Europe, Asia and northwestern Africa. Arabidopsis is popular as a model organism in plant biology, genetics and plant-pathogen interactions. It was chosen as model plant for research, because of ease with which it can be cultivated, its rapid life cycle and high production of seeds (Wixon, 2001). This plant has one of the smallest genomes among plants (125 Mb) and is, therefore the first plant genome to be sequenced in 2000 by the Arabidopsis Genome Initiative (Wixon, 2001; Micali et al., 2008).

Knepper and Day (2010) mentioned that a parallel is drawn between research advances in humans and those that can be directly attributed to studies first conducted in the model plant Arabidopsis. In this sense, around 70% of the genes related with the development of cancer in humans have orthologs present in Arabidopsis. Furthermore, innate immune receptor identification in Arabidopsis plants have made significant impacts in the understanding of disease signaling in human. Resistance proteins were for example first identified and characterized in Arabidopsis before their counterparts in humans (Knepper and Day, 2010)

The scientific progress made on Arabidopsis pathology has gain popularity; particularly, the study of obligate biotrophic and hemibiotrophic pathogens such as mildews, bacteria and nematodes and has significantly contributed to our knowledge regarding the molecular basis of plant defense mechanisms (Sijmons et al., 1991; Katagiri et al. 2002; Micali et al. 2008).
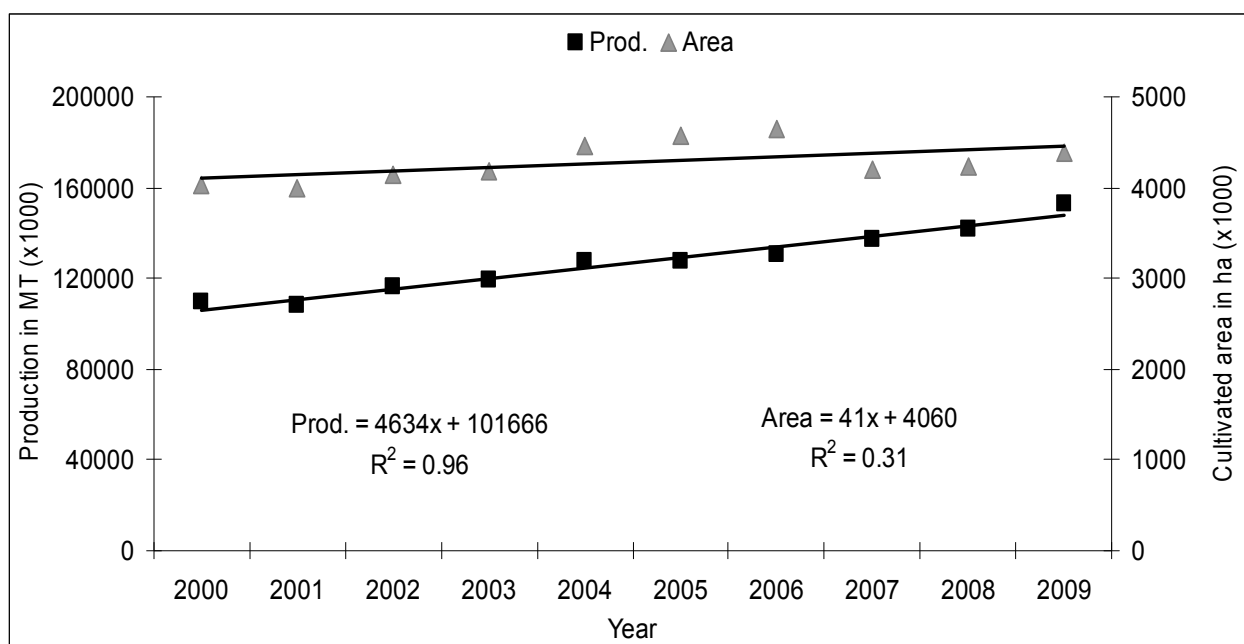
Figure 1.1. Total cultivated area and production of tomato worldwide from 2000 to 2009 and its regression curve. Data from FAOSTAT.
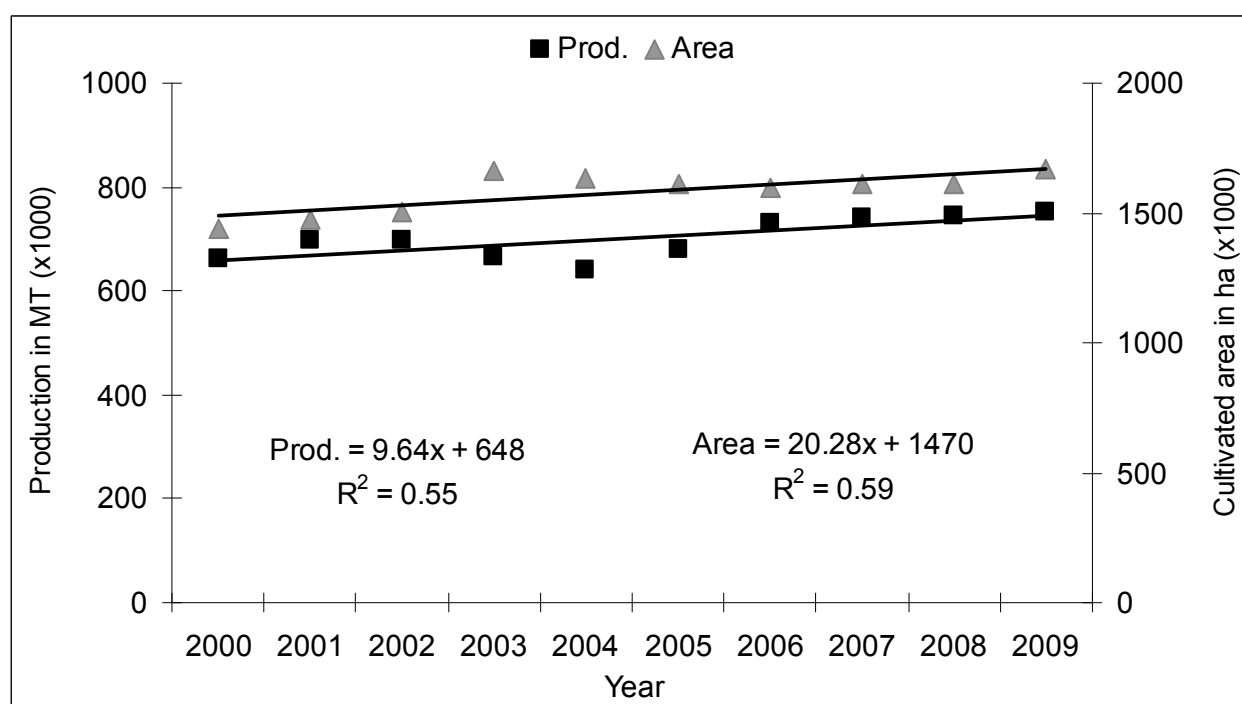
Figure 1.2. Total cultivated area and production of pumpkins, squash and gourds worldwide from 2000 to 2009 and its regression curve. Data from FAOSTAT.

1.2. *Meloidogyne incognita* **and its management**

Root-knot nematodes are the most economically important group of plant parasitic nematodes worldwide, reducing both yield and crop quality (Sasser and Freckman, 1987; Moens et al., 2009). This group of nematodes parasitizes over 2000 plants species and have a highly specialized and complex feeding relationship with their host (Hussey and Janssen, 2002). Plant roots damaged by nematodes become susceptible to soil-borne pathogens. This interaction results in increased plant damages due to the resulting synergistic disease complexes (Sikora and Carter, 1987; Dong et al., 2009; Manzanilla-López and Starr, 2009).

Four species, *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, are the most important species to agriculture. Of these, *M. incognita* is the most economically important species. This nematode is responsible for an estimated average crop loss of 5% around the world and is one of the major obstacles to crop production in many developing countries (Hussey and Janssen, 2002). The life cycle of *Meloidogyne* spp. consists of five stages. Embryonic development that occurs in the egg leads to the formation of the vermiform first stage juvenile (J1), which later moults inside the egg into the second-stage juvenile (J2). The J2 hatches by breaking through the egg shell, at which time it becomes the only infective stage. All other stages take place inside root tissue (Abad et al., 2009). Hatching of *Meloidogyne* is temperature depended and occurs without requiring stimulus from plant root exudates. Nevertheless, exudates have been shown to stimulate hatching in some instances (Karssen and Moens, 2006; Ploeg and Maris, 1999; Curtis et al., 2009).

Infective J2 migrate through the soil and are attracted to roots, penetrating behind the root tip. The J2 migrate intercellularly through the cortical tissue towards the differentiating vascular cylinder. The nematode injects secretory proteins produced in their oesophageal gland cells via the stylet into five to seven undifferentiated procambial cells to transform them into specialized feeding cells known as giant cells, becoming the nematode permanent feeding site (Bird, 1962; Jung and Wyss, 1999; Hussey and Janssen, 2002; Karssen and Moens, 2006; Abad et al., 2009). Root tissue surrounding the nematode feeding site undergoes hyperplasia or hypertrophy, originating the galls or root-knots. The juveniles continue developing in the

root, the J2 moults to the third-stage juvenile (J3), then to the fourth-stage juvenile (J4) and finally to the adult stage (Moens et al., 2009). The vermiform males leave the root, while the females develop into a pear shaped adult (Manzanilla-López, 2004). *Meloidogyne* females reproduce using mitotic parthenogenesis. Their eggs are deposited in a gelatinous matrix, known as egg-mass, on the outer surface of the galled roots (Hussey and Janssen, 2002). Starr (1993) reported a mean of 770 ± 190 eggs per egg-mass of *M. incognita* on cotton.

Galled roots are the primary symptom related to root-knot nematodes infection. The size and form of the gall depends on the species implicated, the number of nematodes in the root, host and plant age. The galls interfere with normal root function, reducing water and nutrient uptake; consequently, leading to stunting, wilting and growth inhibition (Sikora and Fernandez, 2005). Root-knot nematodes are managed by means of different strategies, such as cultural, biological and chemical measures. These measures of control include crop rotation, resistant varieties, flooding, fallow, incorporation of organic matter, soil solarization, steam heating, biological control and nematicides (Noling and Becker, 1994; Manzanilla-Lopez, 2004; Sikora et al., 2005). Nematicides are still one of the primary control measures for nematodes. These chemicals are highly effective when use properly, but do not always kill the nematode in the soil. Nevertheless, the use of nematicides has been restricted and many removed from the market due to their potential negative effects on the environment, human health and in some cases due to a reduction of their effectiveness by biodegradation. Therefore, there is a necessity to develop new, safe and effective alternative measures of nematode control (Zuckerman and Esnard, 1994).

1.3. *Aphis gossypii* and its management

Sucking insects are specialized in their mode of feeding (Jones, 2003). While herbivores cause extensive damage to their host plants, sucking insects such as aphids cause modest damage to their host plants (Walling, 2008). Sucking insects pose important challenges to plants, since they reduce sucrose and amino acids produced in leaf, which normally are transported to the shoot, roots and seeds (Winter et al., 1992). This group of insects contains many vectors of viruses and also are known to introduce chemical and/or protein effectors

that alter plant defense signaling, as well as normal plant development (Kaloshian and Walling, 2005). The interrelationship between sucking insects and the host plant is more analogous to a plant biotrophic pathogen interaction, in which the pathogen is sustained in a localized area and is dependent on living cells (Puterka and Burton, 1991; Zarate et al., 2007). This group of insects causes important losses to agriculture due to their broad host range, high reproductive rate, highly evolved feeding strategies, the ability to adapt to a wide range of habitats as well as the emergence of insecticide resistant strains (Goggin, 2007).

Aphis gossypii Glover (Homoptera: Aphididae), commonly known as the cotton or melon aphid, is widely distributed around the world, infesting over 900 plant species (Margaritopoulos et al., 2009). This aphid is a serious problem of field and greenhouse crops, particularly cotton, cucurbitaceous and ornamental plants, since it transmits more than 50 plant viruses as well as causing stunting, discoloration and deformation of host plants (Margaritopoulos et al., 2009; Sadeghi et al., 2009).

A. gossypii is polymorphic with significant variation in both size and color (Rosenheim et al., 1995). Its live cycle is complex, involving sexual and asexual (parthenogenetic) reproduction (Minks and Harrewijn, 1986). A major form of aphid polyphenism is the switch between viviparous parthenogenesis and sexual reproduction by eggs, depending on environmental conditions. Normally, aphids reproduce during the entire year by viviparous parthenogenesis. A parthenogenetic female is able to produce approximately 120 genetically identical embryos in 10 days (Tagu et al., 2008). Both winged and wingless (alate and apterous) individuals are produced and multiple generations exist on the same host plant. Some aphids require regular alternation of plant hosts where sexual morphs mate and lay eggs on the primary host for several generations and give rise to a generation of alate morphs that move to a secondary host, which is usually a different host plant. On the secondary host, the parthenogenic mode of reproduction is initiated and used for subsequent generations (Kaloshian and Walling, 2005).

Aphids can be managed by chemical, biological and/or integrated method. Biological methods include the use of entomopathogenic microorganisms, as well as those that are

mutualistic endophytes. Biorational compounds are also used in control and they are considered compatible with integrated pest management. These compounds include various classes of insect growth regulators, microbial products and synthetic molecules with novel modes of action and plant derived compounds (Horowitz and Ishaaya, 2004; Sadeghi et al., 2009). Also the application of pheromones and natural enemies has been shown to be important (Steinkraus et al., 2002; Adachi et al., 2009). Chemical methods include important groups of insecticides such as organophosphates, carbamates and pyrethroids. These groups of insecticides have a long history of use. However, over time resistance to them has become a serious problem to farmers. They also negatively impact beneficial insects and the environment if used at the wrong times and improperly applies (Després et al., 2007; Sadeghi et al. 2009). Because of these known side-effects, there is an increasing need to develop new, safe and effective alternative aphid control methods.

1.5. Mutualistic endophytic microorganisms as biocontrol agents

The term endophyte was coined by Heinrich Anton De Bary in 1884 and it is referred to fungi or bacteria that colonize internal plant tissues without causing any apparent symptoms to the host plant (Petrini, 1991; Wilson, 1995). Fungal and bacterial endophytes have been isolated from a great number of plants, such as wheat (Larran et al., 2002), bananas (Pocasangre et al., 2000), soybeans (Larran et al., 2002), potato (Racke and Sikora, 1992; Sturz et al., 1999) and tomato (Hallman and Sikora 1994).

Numerous studies have shown that fungi (Kerry, 1987; Whitehead, 1998; Meyer, et al., 2000; Kiewnik and Sikora, 2004) and bacteria (Hallmann, 2001; Siddiqui and Shaukat, 2004; Reimann et al., 2008) applied as biocontrol agents on soil infested with nematodes reduce their negative impact to the host plant. Among economically important nematodes that have been targeted for biological control are *M. incognita*, *Rotylenchulus reniformis*, *Globodera pallida* and *Radopholus similis* (Sikora et al., 2008). Other fungal antagonists also have been used as biocontrol agents: *Arthrobotrys irregularis*, *Paecilomyces lilacinus*, *Pochonia chlamydosporia, Trichoderma* spp. and *Fusarium oxysporum* that infect or prey on nematodes in the soil. A number of endophytic bacteria are also important in regulating

nematode populations: species of *Pseudomonas*, *Bacillus* and *Rhizobium* have been shown to have antagonistic activity (Hasky-Günther et al., 1998; Sikora et al., 2007; Chaves et al., 2009).

The mechanisms of action responsible for nematode biocontrol mediated by endophytes are variable and include: the production of toxic secondary metabolites, competitive exclusion, competition for nutrients, predation, production of repellent compounds, alteration of root exudates, the induction of systemic resistance or a combination of these elements (Becker et al., 1988; Kerry, 1990; Leeman et al. 1995; van Loon et al., 1998; Sturz et al., 1999; Siddiqui and Shaukat, 2003; Kiewnick and Sikora, 2006; Sikora et al., 2007).

Several reports have shown that endophytes provide protection against insects (Breen, 1994; Clement et al., 1997, Akello et al., 2009). The most studied endophytes affecting insects are grass endophytes in the genus *Neotyphodium* (Clavisipitaceae), formerly classified as *Acremonium* (Azevedo et al., 2000; Vega, 2008). Breen (1994) reported control activity on 23 species of insects in 10 families distributed within 5 orders associated to *Acremomium*. This group of endophytes are obligate seed-borne fungi that colonize the aerial parts of grasses (Breen, 1994). Consequently, the control effect toward insects has been related to allelochemical(s) production. Therefore, the greatest activity takes place against foliar feeding insects, especially when concentration of the fungus and associated allelochemicals are high (Breen, 1993, 1994).

The roll of mycorrhizal fungi in control of nematodes and insects also has been reported (Saleh and Sikora, 1984; Diedhiou et al., 2003; Elsen et al., 2008; Gehring and Bennett, 2009; Vannette and Hunter, 2009). Positive, neutral and negative effects of mycorrhiza colonization on performance of insects have been reported (Rieske, 2001; Barker et al., 2005; Kempel et al., 2010) and on nematodes positive bioprotectional effects have been observed (Diedhiou et al., 2003; de la Peña et al. 2006; Sikora et al., 2007; Elsen et al., 2008). However, the use of this group of obligate symbiotic fungi was not readily adaptable to field conditions, because there are obligate symbionts that can only be produced commercially in large densities on living plants at high cost (Sikora et al., 2007).

1.5.1. Non-pathogenic *Fusarium oxysporum*

F. oxysporum is a common and diverse species among soil fungi in cultivated soil around the world. It survives long periods of time as chlamydospores and grows on organic matter as well as in the ectorhiza and endorhiza of many plant species. This group of fungi includes a number of important plant pathogens that negatively affect economically important crops in particular wilt fungi. The majority of *F. oxysporum* isolates, however, are saprophytes and some also have been isolated from healthy roots and shown to be non-pathogenic to plants. These isolates are interesting for pest and disease management studies, since some of them are able to induce resistance against nematodes, fungi and/or insects (Alabouvette et al., 1998; Trouvelot et al., 2002; Sikora et al., 2007; Martinuz, 2010; Menjivar et al. 2011a,b).

Research has shown that non-pathogenic isolates of *F. oxysporum* reduced significantly the number of root-knot nematodes that penetrated, produced galls, and egg masses on biologically enhanced plants (Hallmann and Sikora, 1994; Dababat and Sikora, 2007; Sikora et al., 2008). Similar results were reported in banana when non-pathogenic *F. oxysporum* isolates were tested against *Radopholus similis* (Pocasangre et al., 2000; Vu et al., 2006; Mendoza and Sikora, 2008; Chaves et al., 2009). Martinuz and Sikora (2010), Menjivar (2010) and Menjivar et al. (2011a) observed reduced population development of *A. gossypii* and changes in host choice of *Trialeurodes vaporariorum* when the insects were exposed to squash and tomato plants inoculated with *F. oxysporum.*

1.5.2. Arbuscular mycorrhizal fungi

Between 70 to 90% of land plant species form arbuscular mycorrhiza (Smith and Read, 2008). Thus, the symbiosis of plants and mycorrhiza is of major importance for all terrestrial ecosystems (Schüßler et al., 2001). Research has demonstrated that the mycorrhizal fungus , *Glomus intraradices* is the most frequently used member of the *Glomeromycota.* In this respect, Stockinger et al. (2009) indicated that more than 1200 publications refer to this species.

The effect of *Glomus* spp.towards nematodes has been previously reported (Elsen et al., 2003; Elsen et al., 2008; Reimann et al., 2008); however, with a few exceptions little is known about the influence of this genus against insects (Wurst et al., 2004; Kempel et al., 2010). In addition, conflicting results have being obtained in that there are studies that demonstrated no effect and others enhancement of insect performance after treating plants with *Glomus* spp. (Gange et al., 1999; Vicari et al., 2002; Curriea et al., 2011).

1.5.3. Endophytic bacteria

Hallmann et al. (1998) defined endophytic bacteria as bacteria that can be isolated from surface-disinfected plant tissues, without causing visible harm to the plant. *Pseudomonas* spp. and *Rhizobium etli* are two of the most studied endophytic bacteria towards nematodes. *R. etli* G12, initially identified as *Agrobacterium radiobacter*, was originally isolated from the rhizosphere of potatoes and was initially shown to reduce *Globodera pallida* infection (Racke and Sikora, 1992). The ability of this bacterium to suppress early infection by *M. incognita* and *G. pallida* has been demonstrated (Racke and Sikora, 1992; Hasky-Günther et al., 1998; Hallmann et al., 2001; Reimann et al., 2008).

Lipopolysaccharides, short-chain sugar molecules in the outer cell wall membrane of the bacterium, were identified as an inducing agent of induced resistance (Reitz et al., 2000) and the mechanism involved in resistance development seems to be directly related to nematode recognition and penetration of the root (Reitz et al., 2001). *R. etli* was recently reported to induce resistance towards *T. vaporariorum* and *A. gossypii* on squash plats (Martinuz and Sikora, 2010) and also against Fusarium wilt (Mwangi et al., 2002; Mwangi et al., 2008).

1.6. Combination of endophytic microorganisms as a biocontrol strategy

The variation in the level of biocontrol, mediated by biocontrol agents, is considered a serious disadvantage for practical applications. This lack of consistency may be caused by different factors, like the level of colonization by the biocontrol endophyte and the fact that these biological strategies are normally based on the application of a single microorganism. Fluctuations in biotic and abiotic conditions may also limit the colonization efficacy of a

biological agent in space and time (Dunne et al., 1998). Consequently, co-inoculation strategies, in which different microorganisms with different mechanisms of action are combined, have been suggested to enhance biocontrol activity (El-Tarabily et al., 2000; Reimann et al., 2008; Chaves et al., 2009; Sikora et al., 2010).

Combining biocontrol agents could result in additive level of protection against nematodes and insects as indicated in previous works (Gadelhak et al., 2005; Mendoza and Sikora, 2008; Chaves et al. 2009; Reimann et al., 2008). Whether synergistic control levels can be reached has to date not been shown. Nevertheless, their mutual direct and indirect interactions should be taken into account when combining biocontrol agents, since the concomitant inoculation could result in improved, neutral or even reduced levels of biocontrol. For instance, combined application of *T. harzianum* and arbuscular mycorrhizal fungi (AMF) inhibited the AMF development and colonization (Wyss et al., 1992), whereas AMF establishment was enhanced in presence of another isolate of the same fungus (Filion et al., 1999). In addition, other studies demonstrated that the co-inoculation of AMF and G12 in biocontrol of *M. incognita* led to an additive level of the nematode biocontrol (Reimann et al., 2008). The co-application of *F. oxysporum* and *G. corantum*, on the other hand, did not enhance the biocontol of *M. incognita* (Diedhiou et al., 2003). *F. oxysporum* and *R. etli* when co-inoculated also did not additively or synergistically improve the control of *A. gossypii* or *M. incognita* (Martinuz and Sikora, 2010). Detailed characterization of the defense mechanisms triggered by the various biocontrol agents when simultaneously inoculated and knowledge of the sensitivity of the individual biocontrol agents to another or to specific defense mechanisms would help in making predictions about compatibility and increased efficacy when they are used in combination for enhanced biocontrol.

1.7. Scope of the study

The present *in vivo* and *in vitro* study was initiated to investigate the interrelationship between the endophytic microorganisms *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and *Rhizobium etli* strain G12 when applied together on infection of the root-knot nematode, *Meloidogyne incognita* and the sucking insect *Aphis gossypii* (Hom., Aphididae).

The objectives of these investigations were to determine the:

1. Biological control activity of single and combined application of *F. oxysporum* Fo162, *G. intraradices* 510 and *R. etli* G12 on *M. incognita* infection of tomato.

2. Biological control activity of single and combined application of *F. oxysporum* Fo162, *G. intraradices* 510 and *R. etli* G12 on *A. gossypii* (Hom., Aphididae) in squash.

3. *In vitro* and *in vivo* interactions between *F. oxysporum* Fo162 and *R. etli* G12.

4. Ability of *F. oxysporum* Fo162 to enhance plant growth and to depress *M. incognita* infection in the model plant *Arabidopsis thalian*a.

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Chapter 2
General materials and methods

General materials and methods used in this study are described in this chapter. Additional techniques and procedures applied in individual experiments are described within the respective chapter.

2.1. Biocontrol agents

2.1.1. Origin and culture of *Fusarium oxysporum* strain Fo162

The mutualistic endophyte *F. oxysporum* strain Fo162 was originally isolated from the cortical tissue of surface sterilized tomato root, *Lycopersicon esculentum* Mill. cv. "Moneymarker" in Kenya by Hallmann and Sikora (1994). The fungus was stored at -80 °C using cryo vials (Cryobank^{TM}, Master Group, Merseyside, UK). For production of the fungal inoculum for all experiments, a single frozen pellet was transferred onto Potato Dextrose Agar (PDA) plates (Difco, Sparks, MD, USA) supplemented with 150 mg 1^{-1} of streptomycin sulfate and chloramphenicol to avoid bacterial contamination. The fungal culture was incubated for 3 weeks at 25 ºC in darkness. Then, the mycelium and conidia formed were scraped from the media surface with a spatula and suspended in autoclaved water. Spores were separated from the mycelium by sieving the content through four layers of fine sterile cheese-cloth. Finally, spore density was determined using an hemacytometer (Thomas Scientific, Philadelphia PA) and then adjusted to 10^6 spores g^{-1} substrate with autoclaved water. In all experiments that include the fungus, plants were inoculated with 5 ml of spore suspension into three holes in the substrate located 1 cm from each plant.

2.1.2. Origin and propagation of *Glomus intraradices* strain 510

Inoculum of *Glomus intraradices* strain 510 in expanded clay was kindly provided by Dr. H. von Alten, University of Hannover. For all experiments which included mycorrhiza, the inoculum in the expanded clay was incorporated in the soil at a rate of 5% of total substrate volume.

2.1.3. Origin and culture of *Rhizobium etli* strain G12

R. etli strain G12 was originally isolated from the rhizosphere of potatoes (Racke and Sikora 1992) and initially identified as *Agrobacterium radiobacter*, but in 1998 after additional testing was renamed *Rhizobium etli* G12 (Hasky-Güther et al., 1998). The bacterium was stored at -80 ºC in cryo vials (Cryobank^{TM}, Master Group, Merseyside, UK). For production of the bacterial inoculum, pellets containing the bacteria were transferred from cryo vials onto plates containing solid King's B medium and incubated for 36 h at 28 ºC (King et al., 1954). A loop of bacteria was then transferred from the pre-culture into an Erlenmayer flask containing 100 ml of liquid King's B medium. The bacterium was cultured for 36 h at 28 ºC on a rotary shaker at 100 rpm. This suspension was then centrifuge at 5000 g for 20 min at 10 ºC. The resulting pellet was re-suspended in sterile ¼ concentrated Ringer solution (Merk) and the concentration was adjusted to an optical density of 2 at 560 nm ($OD_{560} = 2$). This concentration represents approximately 1.2×10^{10} cfu ml^{-1} (Hasky-Günther, 1996; Reitz et al., 2000). In all experiments that include the bacterium inoculum, plants were inoculated with 5 ml of bacterial suspension by drenching the substrate surface. Control plants were treated with the same volume of tap water.

2.2. Nematode

2.2.1. Origin and culture of *Meloidogyne incognita*

The root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood race 3 was originally isolated from an infested field in Florida, USA. The nematode was kindly provided by Dr. D. Dickson, University of Florida, Gainsville, USA and was maintained in a box (150 x 80 x 40 cm) filled with sandy loam and permanently cultivated with the susceptible tomato cultivar Furore (*Lycopersicon esculentum* Mill.) in a greenhouse at 27 ± 5 °C. Nematode eggs were extracted from 2 months old heavily galled tomato roots, using the modified extraction technique of Hussey and Barker (1973). Roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochloride (NaOCl) was added to obtain a final

concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was then thoroughly washed with tap water through a sieve combination 250, 100, 45 and 25 μm mesh to remove the NaOCl. Eggs were collected on the 25 μm sieve and then transferred to a glass bottle. The egg suspension was supplied with oxygen from an aquarium pump over 10 days to induce juvenile hatching. To separate active J2 from unhatched eggs or dead J2, a modified Baermann technique over 24 h was used (Oostenbrink, 1960). The collected active J2 were adjusted to 1000 J2 5 ml^{-1} and used immediately as inoculum.

2.3. Insect

2.3.1. Origin and reproduction of *Aphis gossypii* Glover

The initial *Aphis gossypii* Glover (Homoptera: Aphididae) colony was initiated with aphids obtained from Bayer Crop Science (Bayercode: APHIGO, Bayer CropSeience Deutschland GmbH, Langenfeld) in January 2000. For all experiments, with this insect, apterous adults were collected from a colony maintained on cotton (*Gossypium hirsutum* cv Cukurva 1518) in an incubator at 25 ± 2ºC with relative humidity of 60%, light intensity of 2000 Lux and light/dark photoperiod of 16 h/8h.

2.4. Plants used for experiments

2.4.1. Treatment of seeds

Seeds of tomato cv. Moneymaker (*Lycopersicon esculentum* Mill.) and seeds of squash cv. Eight Ball (*Cucurbita pepo*) were surface sterilized by first shaking them in a 75% Ethanol solution for 1 min and then in a 1.5% Sodium hypochloride (NaOCl) solution for 3 min. Subsequently, the seeds were washed with autoclaved water and transferred to autoclaved sand for germination as described below.

Seeds of *Arabidopsis thaliana* wild type Columbia were surface sterilized as described above. For experiments conducted under sterile conditions, seeds were transferred into sterile Petri dishes on 2% Knop medium, as previously described by Sijmons et al. (1991) or autoclaved sand. For experiments conducted in a climatic chamber, seeds were sown in 4 cm diameter

pots containing autoclaved sand. The seeded Petri dishes or pots held at 4 °C for 3 days prior to incubation in a climatic chamber as described below.

2.4.2. Tomato plants

Sterile seeds of tomato cv. Moneymaker (*Lycopersicon esculentum* Mill.) were sown in 96-well multi-pot trays (50.5 x 30 x 5 cm) each containing approximately 1 kg of autoclaved sand passed through a 2 mm mesh screen. The trays were maintained in a climatic chamber at 25 ± 3 °C with 16 h day^{-1} supplemental artificial light. The tomato seedlings were fertilized with 5 ml of a commercial fertilizer (14-10-14, 2 g l^{-1}) (AGLUKON, Düsseldof, Germany) as needed. After 2 weeks the seedlings were considered ready for experiments.

2.4.3. Squash plants

Sterile seeds of squash cv. Eight Ball (*Cucurbita pepo*) were sown in 70-plug commercial seedlings trays containing approximately 800 g of autoclaved sand passed through a 2 mm mesh screen. The trays were maintained in a climatic chamber at 25 ± 3 °C with 16 h day^{-1} supplemental artificial light. Seedlings were fertilized with 5 ml of a commercial fertilizer (14-10-14, 2 g l^{-1}) (AGLUKON, Düsseldof, Germany) as needed. After 2 weeks the seedlings were considered ready for experiments.

2.4.4. *Arabidopsis thaliana* plants

Experiments under sterile condition: Sterile seeds of *A. thaliana* wild type Columbia were sown in Petri dishes on 2% Knop medium (Sijmons et al., 1991). The dishes containing 5 seeds plate^{-1} were incubated in a climatic chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light. After 2 weeks the seedlings were considered ready for experiments.

Experiments under climatic chamber condition: sterile seeds of *A. thaliana* wild type Columbia were sown in 4 cm diameter pots containing 50 g of a mixture of autoclaved sand:soil, 3:1, v/v. The seedlings were maintained in a climatic chamber at 25 ± 3 °C with 16 h day^{-1} supplemental artificial light. Seedlings were fertilized with 2 ml of a commercial

fertilizer (14-10-14, 2 g l^{-1}) (AGLUKON, Düsseldof, Germany) once per week. After 2 weeks the seedlings were considered ready for experiments.

2.5. Substrate for experiments

The substrate for all greenhouse experiments consisted of a mixture of field soil (15% clay, 78% silt and 8% sand) and sand in a rate of 1:2 v/v. Before mixing, both the soil and sand were passed through a 2 mm mesh screen. The mixed substrate was placed in plastic bags and autoclaved for 60 min at 121 °C. One week after autoclaving, plastic bags containing the substrate were opened for air drying and stabilization at room temperature for 48 h. After this period the substrate was considered ready for experiments.

2.6. Culture media and reagents

2.6.1. Potato Dextrose Agar (PDA)

Potato Dextrose Broth (Oxoid LTD)	24 g
Agar (AppliChem GmbH)	18 g
Deionized water	1 l
Chloramphenicol	150 ppm
Streptomycin sulfate	150 ppm

2.6.2. Solid King's B media (Fluka)

Peptone	20 g
Heptahydrated Magnesium Sulfate	1.5 g
Potassium Hydrogen Phosphate	1.5 g
Bacteriological Agar	15 g
Glycerol	10 ml
Deionized water	1 l

2.6.4. Fuchsin acid (Merk)

1% fuchsin acid solution:

Fuchsin acid powder	2 g
Tap water	198 ml

Lactic acid solution:

Lactic acid   1750 ml

Glycerine   126 ml

Tap water   124 ml

1 % of the fuchsin acid added to lactic acid solution

2.6.5. Phloxine B (MERCK)

Phloxine B   15 mg

Tap water   1000 ml

2.6.6. Modified Knop medium

The 2 % Knop medium contained (Sijmons et al., 1991): 2.5 mM K^+, 1.27 mM Ca^{2+}, 0.2 mM Mg^{2+}, 2.54 mM NO_3^-, 0.5 mM $H_2PO_4^-$, 0.2 mM SO_4^{2-}, 2 µM Na^{2+}, 1.8 µM Mn^{2+}, 0.14 µM Zn^{2+}, 60 nM Cu^{2+}, 24 nM Co^{2+}, 24 µM Cl^-, 9 µM BO_3^{3-}, and 60 nM MoO_4^{2-}. Fe was added as 20 µM Fe^{3-}-NaEDTA. The pH was adjusted to 6.4 with KOH. Before autoclaving (20 min at 121 °C), 2% (w/v) sucrose, 0.8% Daishin agar (Brunschwig Chemie BV) (w/v), and B5 vitamins (Gamborg's solution) were added.

2.7. Statistical analysis

Data from all experiments were tested for normality and homogeneity of variances and subjected to one-way analysis of variance (ANOVA). The data found to be non-homogenous and/or non-normal were log or square-root transformed before statistical analysis and back transformed after analysis. When the overall F-test was significant, the mean values per treatment were compared using the least significant difference test (LSD) at $P \leq 0.05$. Experiments that had only two treatments were analyzed according to a t-test for comparing two independent samples at $P \leq 0.05$. In Chapter 5, non-lineal regression analysis [$Y(t) = \alpha + (3.6-\alpha)\exp(-\beta*t)$] was used to analyze the *in vitro* effect of *R. etli* G12 incubation period on *F. oxysporum* Fo162 radial growth. All statistical analysis was performed using the statistical software InfoStat/Professional version 2009 (InfoStat Group, FCA., AR).

2.8. References

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Chapter 3

Influence of single or multiple inoculation of tomato with the mututalistic endophytes *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and *Rhizobium etli* strain G12 on *Meloidogyne incognita* infection and development

3.1. Introduction

The control of root-knot nematodes is normally achieved with soil fumigants or systemic nematicides (Masadeh et al., 2004). Due to the loss of these pesticides from the market place and to high toxicity, biological measures of control are urgently being investigated worldwide. The mutualistic endophytic fungi *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and the endophytic bacterium *Rhizobium etli* strain G12 are considered as potential non-chemical alternatives (Hallman et al., 2001; Sikora et al., 2007; Reimann et al., 2008).

Hallmann and Sikora (1994) and Dababat and Sikora (2007) demonstrated that tomato plants biologically enhanced with *F. oxysporum* Fo162 reduced the penetration of the root-knot nematode *Meloidogyne incognita*. Similar results were reported in banana when non-pathogenic *F. oxysporum* isolates were tested against *Radopholus similis* (Vu et al., 2006; Chaves et al., 2009).

Reimann et al. (2008) and Elsen et al. (2008) showed that tomato and banana plants treated with *G. intraradices* reduced effectively *M. incognita* and *R. similis*. Moreover, Mycorrhiza fungi have been shown to be important plant growth and health promoting factor (Saleh and Sikora, 1984; Barea and Jeffries, 1995). Dehne (1982) and Singh et al. (2000) mentioned that mycorrhiza colonization is able to provide protection against a broad range of soil-borne fungal pathogens.

Hasky-Günther and Sikora (1995) and Schäfer et al. (2006) reported the effect of *R. etli* G12 against the sedentary nematodes *Globodera pallida* and *M. incognita*, in potato and tomato respectively. In all cases, the presence of the endophytic bacterium resulted in lower juvenile penetration in the root system.

The variation in the level of biocontrol mediated by endophytes is considered a serious disadvantage for commercial application. This lack of consistency may be caused by different factors such as the level of root colonization by the endophyte and the moderate level of colonization by any one of the antagonist. This is a problem due to the fact that biological control is normally based on the application of a single microorganism. Fluctuations in biotic and abiotic conditions in the soil may also alter root colonization efficacy in space and time (Dunne et al., 1998). Consequently, it has been suggested that multiple inoculation strategies, in which different microorganisms with different mechanisms of action, are used could enhance biocontrol activity (El-Tarabily et al., 2000; Mendoza and Sikora, 2009).

The objectives of these investigations were to:

1. Determine the effect of single and dual applications of *F. oxysporum* strain Fo162 with *G. intraradices* strain 510 or with *R. etli* strain G12 on the penetration of *M. incognita*.

2. Evaluate the influence of dual inoculation with *F. oxysporum* strain Fo162 and *R. etli* strain G12, in a spatially-separated plant bioassay on the early root penetration of *M. incognita*.

3. Determine the effect of single inoculation with *F. oxysporum* strain 162 or *R. etli* strain G12 on the rate of development of *M. incognita* after penetration

3.2. Materials and methods

3.2.1. Plants and substrate

Tomato plants cv. Moneymaker were used in all experiments. Plants were cultivated until ready for experiments as described in Chapter 2, Sections 2.4.1 and 2.4.2. The substrate for all experiments in this study was prepared as indicated in Chapter 2, Section 2.5.

3.2.2. Microorganisms

3.2.2.1. *Fusarium oxysporum* Fo162

F. oxysporum Fo162 (Fo162) was stored at -80 °C using cryo vials (Cryobank^{TM}, Master Group, Merseyside, UK). For production of the fungal inoculum for all experiments, a single frozen pellet was transferred onto Potato Dextrose Agar (PDA) plates (Difco, Sparks, MD, USA) supplemented with 150 mg 1^{-1} of streptomycin sulfate and chloramphenicol to avoid bacterial contamination. The fungal culture was incubated for 3 weeks at 25 ºC in darkness. Then, the mycelium and conidia formed were scraped from the media surface with a spatula and suspended in autoclaved water. Spores were separated from the mycelium by sieving the content through four layers of fine sterile cheese-cloth. Finally, spore density was determined using a hemacytometer (Thomas Scientific, Philadelphia PA) and then adjusted to 10^6 spores g^{-1} substrate with autoclaved water.

3.2.2.2. *Glomus intraradices* 510

Glomus intraradices 510 (AMF) in expanded clay was kindly provided by Dr. H. von Alten, University of Hannover. For all experiment which included mycorrhiza, the inoculum was incorporated in expanded clay at a rate of 5% of total substrate volume.

3.2.2.3. *Rhizobium etli* G12

R. etli G12 (G12) was stored at -80 ºC in cryo vials. For production of the bacterium inoculum, pellets containing bacteria was transferred from cryo vials onto plates containing

solid King's B medium for 36 h at 28 ºC (King et al., 1954). A loop of bacteria was transferred from the pre-culture into an Erlenmayer flask containing 100 ml of liquid King's B medium. The bacterium was cultured for 36 h at 28 ºC on a rotary shaker at 100 rpm. The bacterial suspension was centrifuge at 5000 g for 20 min at 10 ºC. The resulting pellets was re-suspended in sterile ¼ concentrated Ringer-solution (Merk) and the concentration was adjusted to an optical density of 2 at 560 nm ($OD_{560} = 2$).

3.2.2.4. *Meloidogyne incognita*

M. incognita was maintained in a box (150 x 80 x 40 cm) filled with sandy loam and permanently cultivated with the susceptible tomato cultivar Furore (*Lycopersicon esculentum* Mill.) in a greenhouse at 27 ± 5 °C. Nematode eggs were extracted from 2 month old heavily galled tomato roots, using the modified extraction technique of Hussey and Barker (1973). Roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochloride (NaOCl) was added to obtain a final concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was then thoroughly washed with tap water through a sieve combination 250, 100, 45, and 25 μm mesh to remove the NaOCl. Eggs were collected on the 25 μm mesh sieve and then transferred to a glass bottle. The egg suspension was supplied with oxygen from an aquarium pump over 10 days to induce juvenile hatching. To separate active J2 from unhatched eggs or dead J2, a modified Baermann technique over 24 h was used (Oostenbrink, 1960). The collected active J2 were adjusted to 1000 J2/5 ml and used immediately as inoculum.

3.2.3. Bioassays

3.2.3.1. Single and dual application of Fo162 with AMF or G12 on *M. incognita* penetration

To assess whether a co-application of Fo162 with AMF or G12 lead to additive or synergistic biocontrol activity towards *M. incognita*, two bioassays were setup as follow:

Bioassay 1. Two weeks old tomato plants were inoculated with water, Fo162, AMF or Fo162+AMF.

Bioassay 2. Two weeks old tomato plants were inoculated with water, Fo162, G12 or Fo162+G12.

Fo162 was applied with 5 ml of a spore suspension at a concentration of 1 x 10^6 CFU g^{-1} of substrate dispensed over 3 holes around the selected plant base. AMF contained in expanded clay was incorporated at a rate of 5% of total substrate volume. G12 was applied with 5 ml pot^{-1} of a bacterial suspension (OD_{560} = 2) as a drench around the stem base of the selected plant. Fo162 and G12 inoculations were repeated 2 weeks later as previously described by Dababat and Sikora (2007). Four weeks after the first inoculation, all plants were inoculated with a 5 ml tap water suspension containing 1000 J2 $plant^{-1}$. The nematodes were added to three 5 cm deep holes, 1 cm from the plant base.

Eight plants per treatment were used and the experiment was conducted twice. The plants were randomly arranged in a green house at 27 ± 3°C with 16 h day^{-1} diurnal light. Two weeks after nematode inoculation, the number of penetrated nematodes was determined by staining the roots with 0.1% acid fuchsin solution, followed by heating to boiling using a microwave for 1.5 to 2 min (Ferris, 1985; Dababat and Sikora, 2007). After cooling for 60 min, excess acid fuchsin was removed by rinsing the roots with tap water. The roots were then macerated twice for 10 s in 25 ml water at high speed (11000 rpm) using an Ultra Turrax® T25 (Whatman GmbH, Dassel, Germany). The macerated root suspension was adjusted to 100 ml with tap water and thoroughly mixed by shaking. From this, two winding-track counting trays (Hooper et al., 2005) were immediately filled with 10 ml aliquots each. The number of penetrated J2 was then counted under a stereomicroscope (100x magnification) and the total number of J2 per root system calculated.

3.2.3.2. Spatially-separated dual inoculation of Fo162 and G12 on *M. incognita* penetration and endophyte colonization

The shoots of 6-week-old tomato plants were completely detached from their respective root system and the basal part of the shoot split longitudinally into three sections, over 7 cm in length. Each section was replanted in a separate 11 cm pot filled with 300 g of autoclaved substrate (Chapter 2, Section 2.5) and each pot was separated approximately 1-cm to guarantee complete physical separation (Fig. 3.1). The lower leaves were pruned to reduce transpiration during the growth of adventitious roots. The triple-split-rooted plants were maintained in a greenhouse for 2 weeks at $27 \pm 3°C$ with 16 h day-1 diurnal light.

Two sections of the triple-split-root plants were then labeled 'inducer', and the third section was labeled 'responder' (Fig. 4.1). One inducer root section was inoculated with Fo162, and the other inducer section was inoculated with G12. Three controls were included in the experiment, in which both of the inducer sections were inoculated with Fo162, G12, or water. Fo162 was applied as a spore suspension in 5 ml pot^{-1} at a concentration of 1×10^6 CFU g^{-1} of substrate. The inoculum was dispensed into three holes around the selected inducer section and inoculation repeated two weeks later as previously described by Dababat and Sikora (2007). G12 was applied with 5 ml pot^{-1} of a bacterial suspension ($OD_{560} = 2$) as a drench around the inducer section of the plant and inoculation repeated 2 weeks later as described by Reimann et al. (2008). Four weeks after the first inoculation, the responder section of each plant was inoculated with a 5 ml suspension containing 1000 J2. The nematode suspension was dispensed into three 2-cm-deep holes around the responder root section.

Treatments were replicated six times and the experiment was conducted twice. The plants were arranged in a completely randomized design in the same greenhouse at $27 \pm 3°C$ with 16 h day^{-1} diurnal light. Two weeks after nematode inoculation, inducer roots and responder roots were collected separately. Nematode penetration was then determined by staining the responder roots with 0.1% acid fuchsin solution as detailed in Section 3.2.3.1.

To determine Fo162 colonization in the inducer root sections, the roots were surface sterilized by submersion in a 1.5% NaOCl solution for 3 min, followed by three rinses in sterile distilled water. The roots were then imprinted on PDA to verify surface sterilization (Schulz et al., 1999). The surface sterilized inducer roots were then cut into 0.5 cm sections and 18 root segments per treatment were randomly selected and placed on two Petri dishes (150 mm diameter) containing PDA medium (Chapter 2, section 2.6.1). After 4 to 7 days of growth at 25°C in the dark, fungi emerging from each root segment were phenotypically verified as being Fo162. These data were used for calculating the percentage of root colonization per inducer root section. The density of G12 was not determined due to the similarity of the colonies with other root inhabiting microorganisms.
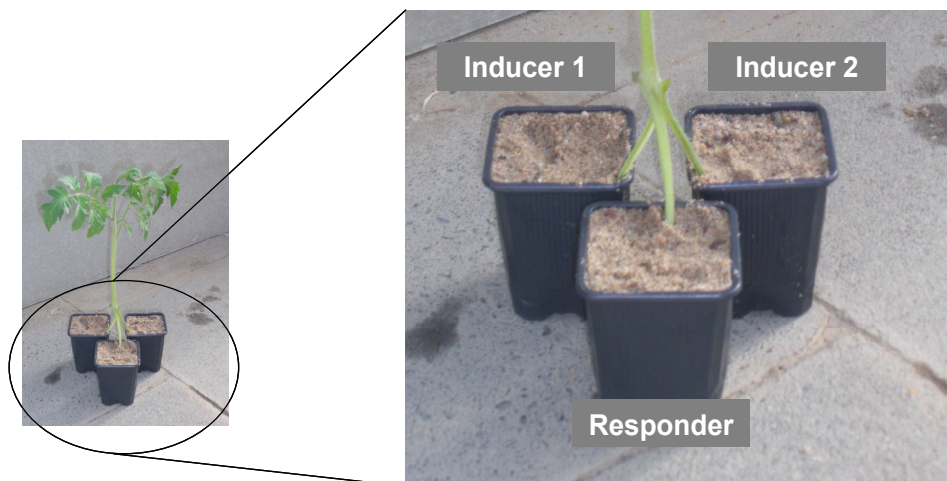
Figure 3.1. Triple-split-root plant designed to study the effect of spatially-separated inoculation of *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* strain G12 on the penetration of *Meloidogyne incognita* on tomato.

3.2.3.3. Influence of Fo162 and G12 on the development of *M. incognita*

To determine the effect of single applications of Fo162 or G12 on the development of *M. incognita*, nematode penetration was synchronized to establish uniformity of development over treatments. Tomato plants were initially inoculated with Fo162 or G12 two and four weeks before transplanting into the experiment pots Plants treated with water serve as controls. Fungal and bacterial density and application techniques were described in section 3.2.3.1. The 4 week old treated plantlets were transplanted into plastic pots filled with 300 g

of substrate. After transplanting, pots were arranged in a completely randomized design on a greenhouse bench at $27 \pm 3°C$ with 16 h day^{-1} supplemental artificial light. One week after transplanting, all plants were inoculated with 5 ml tap water suspension containing 2000 J2 plant^{-1}. The nematodes were added to five 4 cm deep holes, 1 cm from the plant base. Two days after nematode inoculation, the plants were removed from their pots and carefully washed free from substrate and non-penetrated J2. Immediately thereafter, the plants were replanted into new pots containing 800 g of autoclaved substrate. Each treatment was replicated 18 times and the experiments were repeated. After transplanting into the new soil, the pots were arranged in a completely randomized design on a greenhouse bench under the conditions previously indicated.

The developmental stages J2, J3, J4 and adults were determined 14 and 21 days after nematode inoculation. Six plants per treatment and interval were randomly selected and removed from their pots and carefully washed free from substrate. Nematodes were separated from the root tissue by staining and blending (Section 3.2.3.1) and the different stages counted under the microscope. Female fecundity was determined 35 days post nematode inoculation by uprooting 6 plants and gently washing the roots free from substrate. Egg-masses were then stained in 0.015% Phloxine B for 20 minutes (Chapter 2, section 2.6.5), washed with tap water to remove extra stain and then 10 egg masses were randomly selected and placed into a 100 ml graduated cylinder containing 10 ml of 1.5% NaOCl solution for 15 min. The egg suspension was then thoroughly washed with tap water through a 25 μm mesh sieve to remove the NaOCl. Eggs remaining on the sieve were transferred to a 100 ml graduated cylinder and the solution, adjusted to 30 ml with tap water. Eggs in two 2 ml aliquots were counted and total number of eggs per egg-mass extrapolated.

3.3. Results

3.3.1. Single and dual application of Fo162 with AMF or G12 on *M. incognita* penetration

3.3.1.1. Bioassay 1, Fo162 and AMF

Two weeks after nematode inoculation, nematode penetration per root system was significantly reduced in plants inoculated with Fo162 and AMF alone or in combination in both Experiments (Table 3.1). The application of Fo162 or AMF singly decreased nematode penetration 78 and 58 percent in Experiment 1 and 51 and 44 percent in Experiment 2, when compared to the control, respectively. Combined application of Fo162 with AMF caused a slight higher reduction in penetration of 68 and 48 percent in both experiments, when compared to the control, respectively. However, the combined application of Fo162 with AMF did not lead to synergistic or additive levels of reduction in penetration when compared to their individual application (Table 3.1).

Table 3.1. Effect of single and dual application of *Fusarium oxysporum* strain Fo162 (Fo162) and *Glomus intraradices* strain 510 (AMF) on *Meloidogyne incognita* penetration, two weeks after nematode inoculation.

Treatment	Nematodes per root system[1,2]	
	Exp.1	Exp.2
Fo162	18.75 ± 4.79 a	48.75 ± 5.49 a
AMF	36.25 ± 4.60 b	55.00 ± 7.79 a
Fo162+AMF	27.5 ± 4.53 ab	51.25 ± 6.11 a
Control	86.25 ± 12.53 c	98.75 ± 9.15 b
P-value	<0.0001	0.0001
LSD	21.56	21.08

[1]Data are expressed as mean ± standard error; [2]Columns with different letters are significantly different after LSD test ($P \leq 0.05$, n = 8).

3.3.1.2. Bioassay 2, Fo162 and G12

Penetration per root system, assessed two weeks after nematode inoculation, was significantly reduced when the plants were inoculated with Fo162 and G12 alone or

combined in two independent experiments (Table 3.2). Single application of Fo162 or G12 reduced nematode penetration by 57and 53 percent in Experiment 1 and by 39 and 45 percent in Experiment 2, when compared to *M. incognita* inoculated control, respectively. Concomitant inoculation of Fo162 and G12 decreased penetrated nematodes by 46 and 37 percent in both Experiment 1 and 2, when compared to the control, respectively. Nonetheless, the combined application of both Fo162 and G12 did not lead to additive or synergistic levels of reduction in penetration, when compared to their individual effects (Table 3.2).

Table 3.2. Effect of single and dual application of *Fusarium oxysporum* strain Fo162 (Fo162) and *Rhizobium etli* strain G12 (G12) on *Meloidogyne incognita* penetration, two weeks after nematode inoculation.

| Treatments | Nematodes per root system[1,2] | |
	Exp.1	Exp.2
Fo162	30.57 ± 8.37 a	53.38 ± 9.27 a
G12	35.57 ± 12.05 a	48.63 ± 17.88 a
Fo162+G12	41.00 ± 9.06 a	59.75 ± 14.94 a
Control	127.0 ± 15.40 b	131.1 ± 13.94 b
P-value	<0.0001	0.0009
LSD	33.75	41.55

[1]Data are expressed as mean ± standard error; [2]Columns with different letters are significantly different after LSD test ($P \leq 0.05$, n = 8).

3.3.2. Spatially-separated dual inoculation of Fo162 and G12 on *M. incognita* penetration

A triple split-root experiment was used to evaluate the ability of Fo162, G12 or a combination of both endophytes to reduce *M. incognita* penetration when all organisms were separately inoculated (Fig. 3.1). Two weeks after nematode inoculation, *M. incognita* penetration in the responder section of the triple-split-root system was reduced significantly following fungal inducer, bacterial inducer and both fungal and bacterial inducer in Experiment 1 (*P*=0.0006, LSD=24.61) and Experiment 2 (*P*=0.0052, LSD=108.86), when compared to the nematode penetration of control plants treated with water (Fig. 3.2). However, the number of penetrated nematodes detected on those roots treated with Fo162 at one side and G12 at the other (Fig. 3.2; fungal-bacterial inducer) was non-significantly

different when compared to those which were treated with either G12 or Fo162 at both inducer sides in either of the two experiments (Fig. 3.2).

Re-isolation of Fo162 from the Fo162 inoculated section of the root system, in both experiments, revealed a reduction in root colonization of 35% (*P*=0.0164) and 39% (*P*=0.0306) when G12 was inoculated on a separate inducer root section in Experiment 1 and 2, respectively (Fig. 3.3). Fo162 was neither detected in the water control root sections nor in the root sections inoculated with G12.
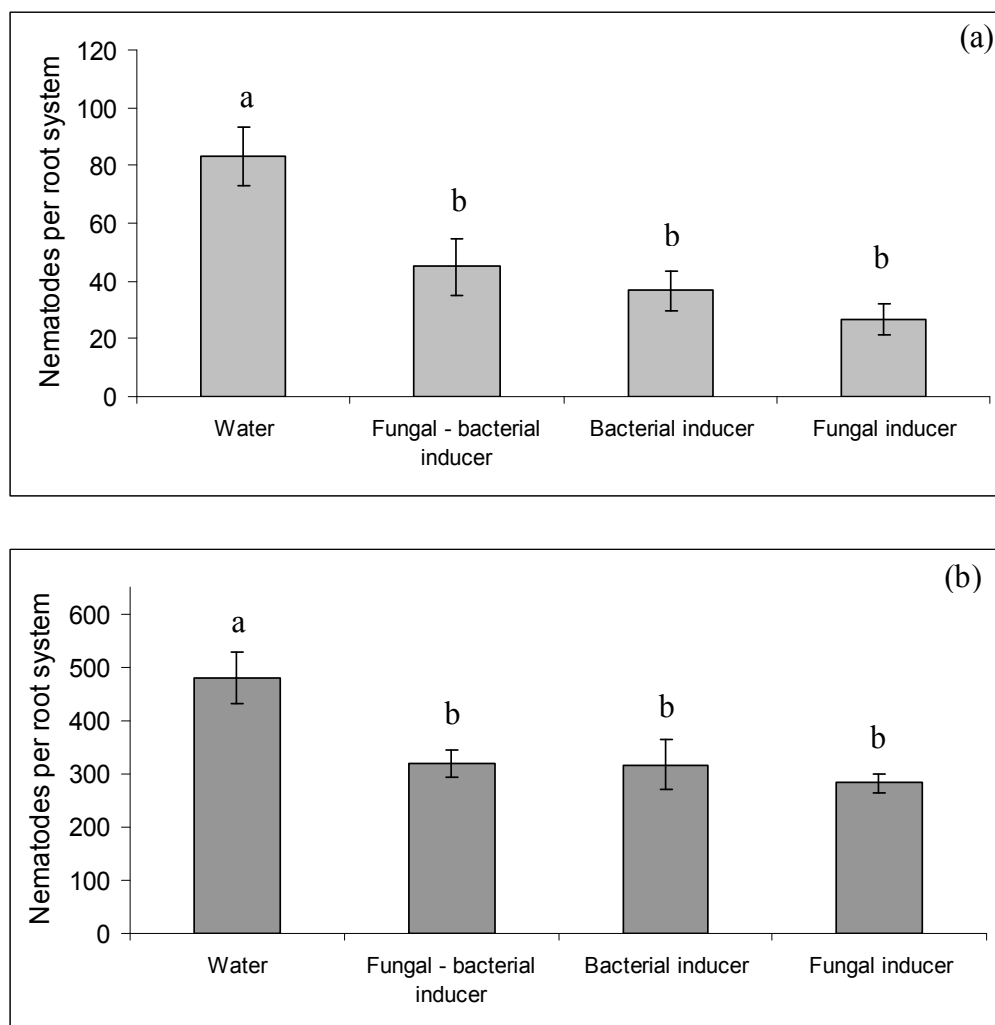
Figure 3.2. Effect of spatially separated co-inoculation of *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* G12 on *Meloidogyne incognita* penetration. Vertical bars represent standard error of the mean values. Columns with different letters are significantly different after LSD test (*P* ≤ 0.05, n = 6). (a) Experiment 1 and (b) Experiment 2.
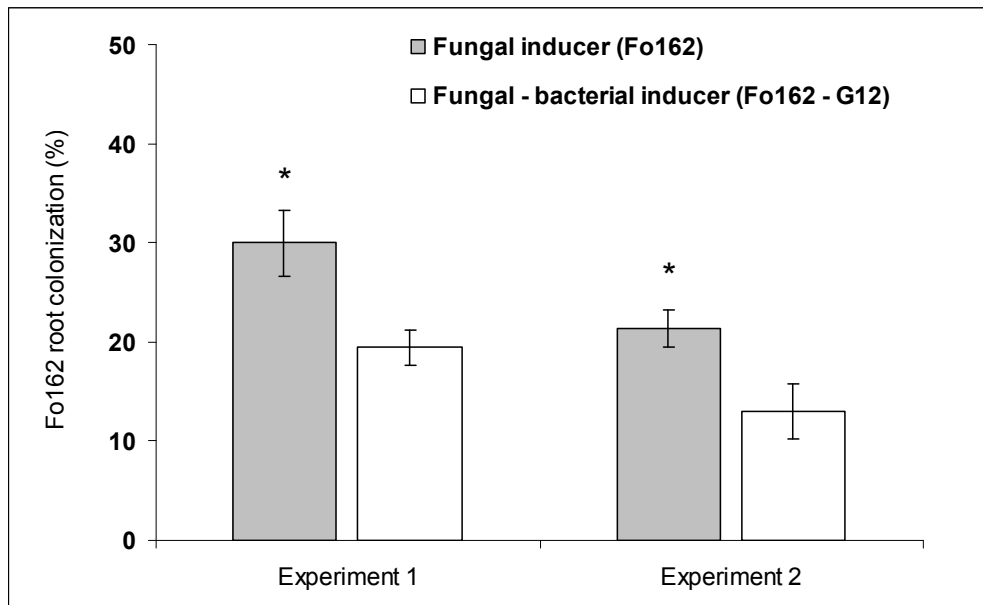
Figure 3.3. Re-isolation of *F. oxysporum* Fo162 (Fo162) from the inducer side of a triple split-root chamber designed to study the systemic activity towards *M. incognita* as induced by spatially-separated inoculation of *F. oxysporum* Fo162 and *R. etli* G12. Vertical bars represent standard error of the mean values. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=6).

3.3.3. Influence of Fo162 and G12 on the development of *M. incognita*

3.3.3.1. Fo162

The influence of Fo162 on *M. incognita* development was assessed after synchronizing the nematode penetration. Fourteen days after nematode inoculation, the number of J2 was significantly higher 114 and 34 percent in Fo162 treated plants than in untreated plants in both experiments (Fig. 3.4 a and b). Conversely, the number of J3 was significantly lower, 48 and 73 percent, in Fo162 treated plants when compared to untreated plants in the two experiments. The number of J4s was reduced 72 percent in the Fo162 treated plants in the first experiment (Fig. 3.4a), but high variability and low numbers of J4s did not yield significant differences. In the second experiment J4 stages were not produced.

The number of J2s that developed to adults was reduced significantly 82 and 69 percent 21 days after nematode inoculation in plants inoculated with Fo162 in both experiments (Fig.

3.4 b and c). The number of J2s that developed to the J4 stage also was reduced, 39 and 31 percent, in the Fo162 treated plants. Conversely, the number of J2 and J3s was higher in plants treated with Fo162 than in untreated plants in both experiments (Fig. 3.4 b and c). Moreover, female fecundity in plants treated with Fo162 was negatively affected. Egg production per female, 35 days after nematode inoculation, was reduced by 59 and 41 percent in both tests. (Fig. 3.4 e and f). There was a highly significant reduction of 40 and 29 percent in the total number of nematodes in the roots of Fo162 treated plants when all developmental stages were added together in both experiments (Fig. 3.5). These results reconfirmed those obtained in the previous experiments presented in Tables 3.1 and 3.2. The results demonstrated high levels of suppression of penetration and delayed nematode development within the root of Fo162 colonized plants.

3.3.3.2. G12

Fourteen days after nematode inoculation, the number of J2 was not significantly different when compared to the untreated control in either experiment (Fig. 3.6 a and b). However, the presence of G12 cased a significant reduction in the number of J3, 35 and 52 percent when compared to controls in both Experiments (Fig. 3.6 a and b). The number of J4s, which were only produced in Experiment 2 were not affected by G12 when compared to the control. Twenty-one days after nematode inoculation, a significant decreased in the number of juveniles that developed to adults was detected, 60 and 38 percent, in both experiments (Fig. 3.6 b and c). J3 and J4 stages developed in both experiments but significant differences were not detected.

Moreover, in plants treated with G12 a significant reduction in fecundity was observed. The number of eggs per female, 35 days after nematode inoculation was significantly reduced 22 percent in the first experiment and non-significantly 14 percent in the second experiment (Fig. 3.6 e and f). Furthermore, overall nematode penetration calculated by adding the different live stages resulted in a significant reduction of 34 and 32 percent in G12 treated plants in both experiments (Fig. 3.7). These results reconfirmed the results obtained with G12 in the experiments on penetration shown in Tables 3.1 and 3.2. Overall the results demonstrated

high levels of G12 inhibition of nematode root penetration and suppression of nematode development within the root after penetration.
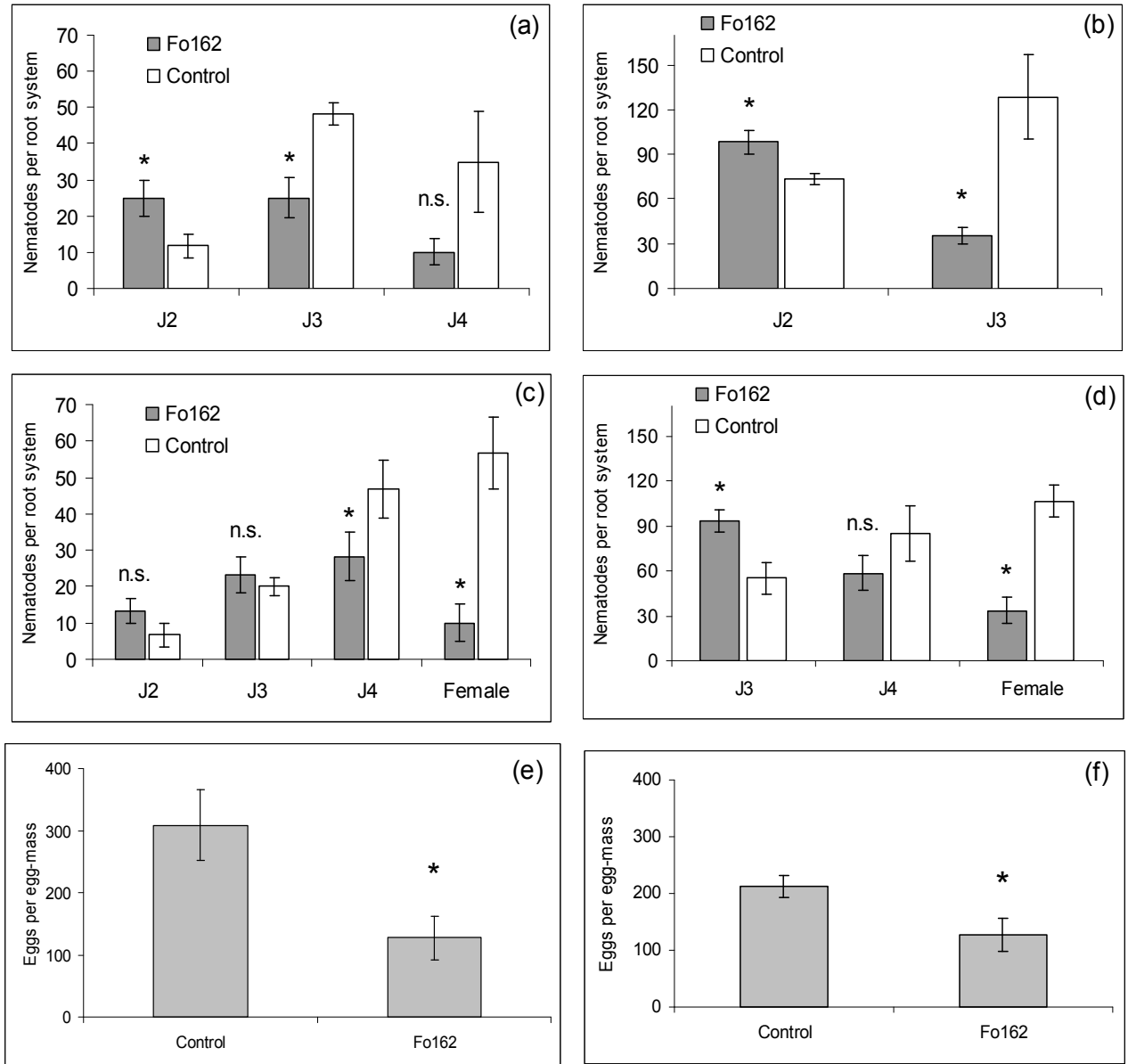
Figure 3.4. Effect of *F. oxysporum* strain Fo162 (Fo162) on the development of the root-knot nematode *M. incognita.* Vertical bars represent standard error of the mean. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=6). Means with 'n.s.' indicates not significantly different. (a) and (b) nematode live stages assessed 14 days post nematode inoculation (dpi) in Experiment 1 and 2 respectively. (c) and (d) nematode live stages determined 21 dpi in Experiment 1 and 2 respectively. (e) and (f) nematode fecundity evaluated 35 dpi in Experiment 1 and 2 respectively.
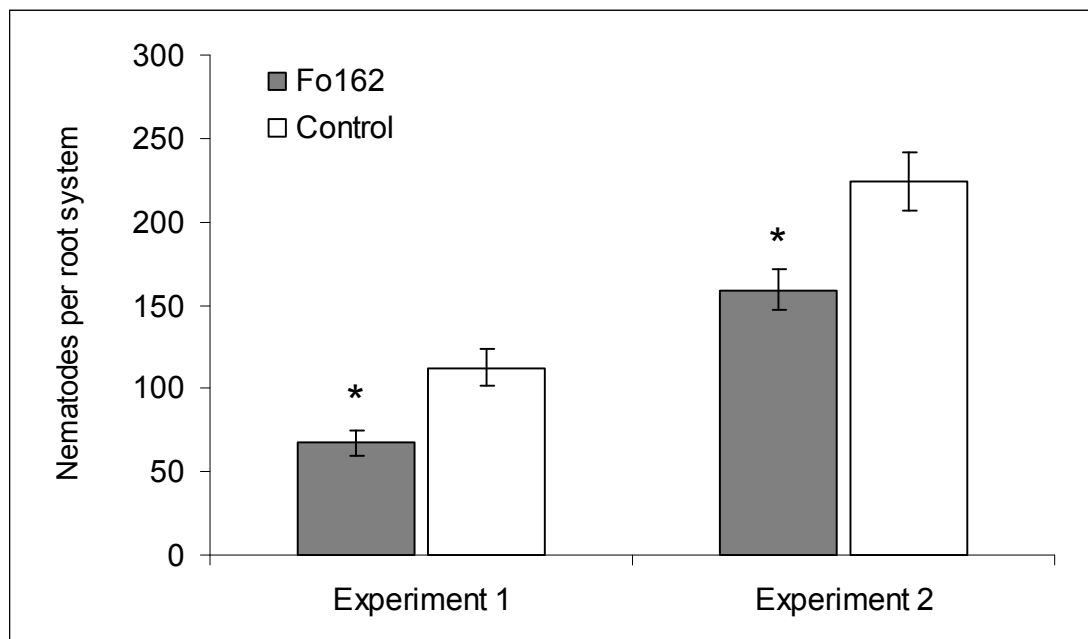
Figure 3.5. *Meloidogyne incognita* penetration in plants treated with *Fusarium oxysporum* strain Fo162 (Fo162) and untreated control plants, after synchronizing nematode penetration. Vertical bars represent standard error of the mean. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=12).

Figure 3.7. *Meloidogyne incognita* penetration of plants treated with *Rhizobium etli* strain G12 (G12) and untreated control plants, after synchronizing the nematode penetration. Vertical bars represent standard error of the mean. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=12).
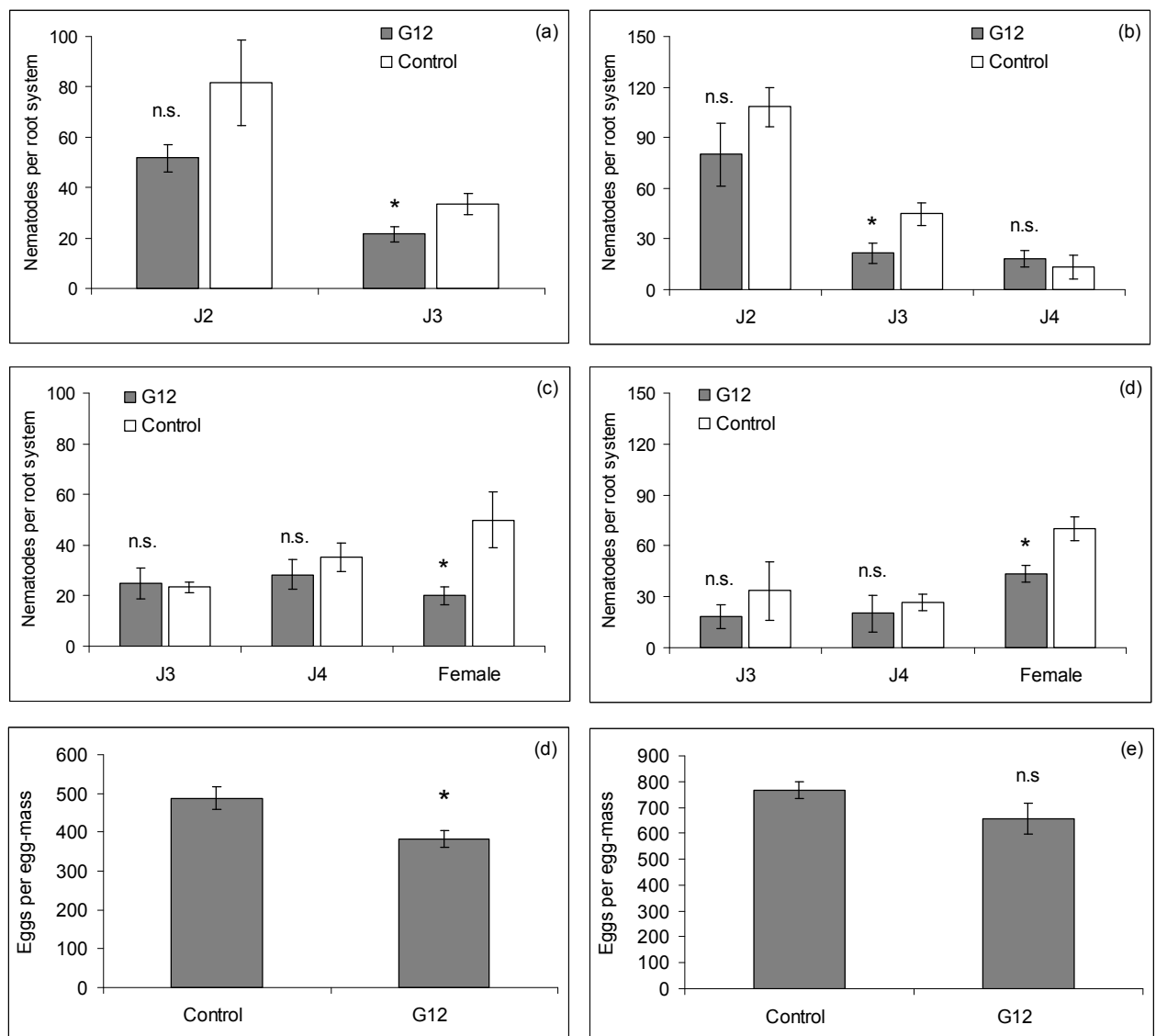
Figure 3.6. Effect of *Rhizobium etli* strain G12 (G12) on the development of the root-knot nematode *M. incognita.* Vertical bars represent standard error of the mean. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=6). Means with 'n.s.' indicates not significantly different. (a) and (b) nematode live stages assessed 14 days post nematode inoculation (dpi) in Experiment 1 and 2 respectively. (c) and (d) nematode live stages determined 21 dpi in Experiment 1 and 2 respectively. (e) and (f) nematode fecundity evaluated 35 dpi in Experiment 1 and 2 respectively.

3.4. Discussion

This study reports on the biocontrol of *M. incognita* by single and combined application of Fo162, AMF and G12. The initial experiments analyzed the ability of the antagonists to reduced *M. incognita* penetration of the root when applied single or combined. In the second set of experiments the ability of Fo162 and G12 to reduce systemically *M. incognita* penetration, when applied in a spatially-separated way to the same plant, was studied. Finally, the influence of Fo162 or G12 alone on nematode development was examined.

Single and dual application of Fo162 with AMF or G12 provided enhanced biological control towards *M. incognita*. However, the combined application of the beneficial organisms did not result in an additive or synergistic effect in reducing penetration. Similar results have been reported by Mendoza and Sikora (2009) when they tested *Paecilomyces lilacinus* and Fo162 in dual tests for activity towards *Radopholus similis*. They attributed the lack of any additive effects to the fact that Fo162 and *P. lilacinus* are applied at different times. This was also the reason that they felt the two antagonists did not interact negatively with each other. Furthermore, Khan et al. (2006) reported similar results when they tested *P. lilacinus* and *Monacrosporium lysipagum* towards different plant parasitic nematodes on different crops. They found that the combined applications of the beneficials were more effective than their single application, but the effect were still not additive or synergistic.

Reimann et al. (2008) demonstrated the effective use of dual application of G12 and AMF for the control of *M. incognita* on tomato. They concluded that specific combinations of plant health promoting bacteria and mycorriza fungi could lead to improved mycorrhizal colonization and improved nematode control. They suggested that the combination of agents with different mechanisms of action was responsible for the higher levels of nematode control. In addition, Chaves et al. (2009) tested the co-application of different endophytic fungi and bacteria to control *R. similis*. They found that combined application of *Tricoderma atroviride* with *Bacillus* or *Pseudomonas* and *F. oxysporum* with *Pseudomonas* led to a higher level of nematode control when compared to their individual application. They concluded that endophytic fungi and bacteria have different modes of action towards the

nematode and that a combination of these mechanisms can lead to a better nematode suppressive efficacy.

In the present studies a triple split-root experiment demonstrated the ability of both Fo162 and G12 to reduce systemically *M. incognita* penetration of tomato. This confirmed previous work regarding the systemic biocontrol activity of each of these two organisms inoculated singly (Hasky-Günther et al., 1998; Vu et al., 2006; Schäfer et al., 2006; Dababat and Sikora, 2007). The simultaneous and spatially-separated application of both beneficial organisms to the individual inducer sides of the triple split-root system also resulted in a significant reduction of *M. incognita* penetration at the responder side of the triple split-root system. However, the level of reduction was not significantly different from the treatments where only one of the biocontrol agents was present. The presence of both microorganisms apparently did not increase the intensity of the systemic resistance response in the plant. Very important was the fact that the presence of G12 at one inducer side, negatively affected the colonization level of Fo162 at the other inducer side.

This indicates that systemic resistance mechanisms initiated by the bacterium not only affect the colonization of the nematode, but also the colonization of Fo162. This was confirmed after re-isolation of the fungus from the inducer sides of a split-root system, showing a reduced colonization of the fungus, when both micro-organisms were simultaneously inoculated in a spatially-separated way in the same plant. These results are to some extent similar to those of Liu et al. (1995) who used a split-root system to study the systemic induced resistance (SIR) produced by two plant growth-promoting rhizobacteria, *Pseudomonas putida* and *Serratia marcescen*, towards Fusarium wilt, caused by *F. oxysporum* f. sp. *cucumerinum*. Both bacteria were able to a delayed disease symptom development and retarded colonization of the pathogen. Moreover, van Peer and Schippers (1992) showed that associated lipopolysaccharides (LPS), short-chain sugar molecules in the outer cell wall membrane of the bacterium, of *Pseudomonas* spp. induce resistance against Fusarium wilt caused by *F. oxysporum* f. sp. *dianthi* in carnation. Apparently, some bacteria are able to induce resistance in plans towards *F. oxysporum* species. It is conceivable that

bacterial induced resistance to species of *Fusarium* is universal and not limited to pathogenic strains as has been shown in the present study.

The results obtained here indicated that some biocontrol agents may be incompatible when colonizing the same plant, thus negatively affecting each other in a direct or indirect way and reducing the biocontrol efficacy. Until now, the type of defense responses initiated by the individual biocontrol agent may be too general to predict the effect of applying two or more biocontrol agents in order to better control a plant disease or pest. Reitz et al. (2000) demonstrated that induced resistance produced by G12 towards cyst nematode was not accompanied by enhanced accumulation of specific PR proteins, such as chitinase and β-1,3-glucanase. In split-root experiments Selim (2010) demonstrated SIR towards *M. incognita* in tomato after treatment of the inducer half with salicylic acid (SA), metyl jasmonate or Fo162. It was concluded that both jasmonic acid (JA)- and SA- dependent signaling pathways are involved in induced resistance against *M. incognita* in tomato, suggesting that both systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the pathways that influence antagonism towards the nematode.

Reduced nematode root penetration could indicate that the root exudates either did not attract or they repelled the J2 from the roots. The lack of attraction of J2 by root exudates produced in Fo162 colonized plants was demonstrated by Dababat (2007) and Dababat and Sikora (2007). They used a linked twin-pot chamber to analyze the effect of Fo162 on *M. incognita* J2 attraction to and penetration of tomato plants. Their results suggested that Fo162 either produces substances that directly repel the nematode, or the fungus alters the root exudates pattern affecting the nematode attraction. In this respect, Selim (2010) analyzed by HPLC root exudates collected from Fo162 treated tomato plants and untreated plants. He found an accumulation of unique compounds in the exudates coming from Fo162 treated plants when compared to control plants; thus, demonstrating alteration in root exudates pattern. Moreover, Vu (2005) combined a split-root system with a twin-pot attraction chamber demonstrating that when the inducer pot of the split-root plant was inoculated with Fo162, the exudates produced in the responder half of the split-root plant was less attractive to *R. similis* than the

exudates produced from the control plant in a third pot attached by a bridge to the responder root segment.

The present experiments with synchronized nematode penetration showed that second-stage juveniles of *M. incognita* that those J2 that are able to penetrated roots of Fo162 and G12 treated tomato plants are negatively affected in the plant during development. Nematode development was retarded in plants enhanced biologically with Fo162 and G12 in comparison to untreated plants. Similar results were obtained by Saleh and Sikora (1984) in plants colonized by *G. fasciculatum*. Egg production was also lower in Fo162 and G12 inoculated plants than in control plants, which indicates competition for nutrients in the root tissue after penetration.

Proite et al. (2008) studied the post-infection development of *M. arenaria* on susceptible and resistant peanut cultivars. They concluded that penetration and development of the nematode in the resistant cultivar was reduced when compared to susceptible ones. The authors reported a hypersensitive-like (HR) response of infested resistance host cells, which occurred 8-19 days after nematode infection; thus, interfering with the normal formation and functioning of the giant cell system. Furthermore, they demonstrated that the nematode development was delayed, since the nematode completed his live cycle in resistant host in 63 days after inoculation, while in susceptible host the nematode completed his live cycle in only 32 days after inoculation. Moreover, HR reactions have been accepted as an important post-infection plant response in nematode resistant cultivars (Moens et al., 2009). To our knowledge, HR has not yet been reported in an interaction between a fungus and a root. The few reports in this respect are related to the case of plant resistant to nematode infection (Williamson and Hussey, 1996; Proite et al., 2008). The observations reported in this study were strictly related to effects of endophyte-treated plans on nematode development, and it would by necessary to perform detailed histological observations of the nematode-endophyte-root interactions to investigate an HR process.

The delayed nematode development could be also related to nutritional competition as a result of the nematode-endophyte interaction. This idea is in line with Chen et al., (2010)

who recently identified a new class of sugar transporters (named SWEETs). They indicated that bacterial symbionts and fungal and bacterial pathogens were capable to induce the expression of SWEET genes, suggesting that the sugar efflux function of SWEET transporters is possible targeted by symbionts and pathogens.

3.5. Conclusions

The biological activity of single or combined application of the antagonists *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and *Rhizobium etli* strain G12 was investigated in this study. From the results the following can be concluded:

1. The individual application of *F. oxysporum*, *G. intraradices* and *R. etli* results in a significant reduction in the number of *M. incognita* that penetrated into tomato roots. However, concomitant enhancement with *F. oxysporum* together with *G. intraradices* or with *R. etli* did not lead to significant synergistic interactions.

2. The simultaneous application of *F. oxysporum* and *R. etli* was tested through a triple split-root experiment. This experiment indicated that the simultaneous but spatially-separated inoculation of both endophytes did not lead to a significant additive effect with respect to reducing *M. incognita* penetration. Furthermore, this co-inoculation showed a significant reduction in root colonization by *F. oxysporum*. This effect demonstrates that the lack of additive or synergism in biocontrol may be due to incompatibility.

3. Single inoculations of *F. oxysporum* and *R. etli* on tomato resulted in a reduction of the number of juveniles that developed to adult females, 14 and 21 days after nematode inoculation, when compared to the untreated control in each time-point respectively. Moreover, fungal and bacterial treated plants showed a significant reduction in number of eggs per adult female, 35 days after nematode inoculation. The data indicate that *F. oxysporum* and *R. etli* lead to retardation in *M. incognita* development and a reduction in fecundity. The mechanism responsible for these effects need further study.

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Chapter 4

Influence of single or multiple inoculation of squash with the mututalistic endophytes *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and *Rhizobium etli* strain G12 on *Aphis gossypii* infection

4.1. Introduction

Aphid control is normally achieved through the used of insecticides belonging to one of the three main pesticide active ingredients: organophosphates, carbamates or pyrethroids. However, due to their long application history, aphid resistance to insecticides has become a serious problem to the growers (Sadeghi et al., 2009). In addition, these pesticides if used improperly can be harmful to human health, natural enemies of aphids and other potentially beneficial insects. These negative side effects strengthen the need for biological alternatives for aphid control. These alternatives include the use of biorational compounds derived from living organisms or the use of classical biocontrol strategies. Biorational compounds are considered compatible with integrated pest management and include various classes of insect growth regulators, microbial based products, synthetic molecules with novel modes of action and plant-derived compounds (Horowitz and Ishaaya, 2004; Sadeghi et al., 2009). Biocontrol strategies include the application of entomopathogenic microorganisms -e.g. *Bauveria bassiana* and *Metarizium anisopliae,* the release of predacious and parasitic beneficial insects, entomopathogenic nematodes and the used of endophytes that directly or indirectly influence insect development and/or behavior.

The best studied endophytes for use in insect management are the grass endophytes in the genus *Neotyphodium* (Clavisipitaceae), formerly classified as *Acremonium* (Azevedo et al., 2000; Vega, 2008). Breen (1994) reported controlling effects of 23 species of insects in 10 families distributed within 5 orders associated with the presence of *Acremomium* in the plant foliar tissue. The endophytes in this group are obligate seed-born fungi, colonizing the aerial parts of grasses (Breen, 1994). The mechanism of insect control associated with these fungi has been related to allelochemical production. Consequently, the efficacy against foliar feeding insects is most effective where the accumulation of hyphae and synthesized allelochemicals are high (Breen, 1993, 1994).

The role of endophytic mycorrhizal fungi towards insects also has been extensively reviewed (Gehring and Bennett, 2009; Vannette and Hunter, 2009). Positive, neutral and negative effects of mycorrhization on the performance and development of insects have been reported (Rieske, 2001; Barker et al., 2005; Kempel et al., 2010; Koricheva et al., 2009). Koricheva et al. (2009) demonstrated by means of a meta-analysis that the density and consumption of chewing insects were higher on mycorrhized plants, although plant damage did not increase. They also reported that sucking insects even benefited from mycorrhizal infection. Mesophyll feeders, however, decreased on mycorrhized plants and sucking insects performed better on plants colonized by endomycorrhiza when compared to those colonized by ectomycorrhiza.

Endophytic fungi and bacteria also can reduce infection of plant pathogens. The fungus *Fusarium oxysporum* strain Fo162 and the bacterium *Rhizobium etli* strain G12, have been shown to increase plant resistance towards root-knot nematodes (Sikora et al., 2007). Both organisms colonize the host ecto- and endorhiza and thereby increased resistance by one or more mechanisms of action: production of toxic secondary metabolites, competitive exclusion, competition for nutrients, the generation of pathogen repelling components in root exudates, the induction of systemic resistance or a combination of these elements (Becker et al., 1988; Kerry, 1990; Leeman et al. 1995; van Loon et al., 1998; Sturz et al., 1999; Siddiqui et al., 2003; Kiewnick and Sikora, 2006; Sikora et al., 2007; Hasky-Günther and Sikora, 1995; Schäfer et al., 2006; Vu et al., 2006; Dababat and Sikora, 2007; Reimann et al., 2008; Sikora et al., 2008). Recent work has shown that the presence of Fo162 and G12 can also reduce colonization by insects (Martinuz, 2010; Menjivar, 2010; Menjivar et al., 2011).

The objectives of the following investigations were to:

1. Determine the effect of single and dual applications of *F. oxysporum* strain Fo162 with *G. intraradices* strain 510 or *R. etli* strain G12 on the performance of *A. gossypii.*

2. Evaluate the influence of dual inoculation with *F. oxysporum* strain Fo162 and *R. etli* strain G12, in a spatially-separated plant bioassay on the performance of *A. gossypii.*

3. Determine the effect of single inoculation with *F. oxysporum* strain Fo162 and *R. etli* strain G12 on host preference of *A. gossypii*, in intact plant choice bioassay.

4.2. Materials and methods

4.2.1. Plants and substrate

Squash plants were used in all experiments. Plants were cultivated until ready for experiments as described in Chapter 2, Sections 2.4.1 and 2.4.3. The substrate for all experiments in this study was prepared as indicated in Chapter 2, Section 2.5.

4.2.2. Organisms

4.2.2.1. *Fusarium oxysporum* Fo162

F. oxysporum Fo162 (Fo162) was stored at -80 °C using cryo vials (Cryobank^{TM}, Master Group, Merseyside, UK). For production of the fungal inoculum for all experiments, a single frozen pellet was transferred onto Potato Dextrose Agar (PDA) plates (Difco, Sparks, MD, USA) supplemented with 150 mg l^{-1} of streptomycin sulfate and chloramphenicol to avoid bacterial contamination. The fungal culture was incubated for 3 weeks at 25 ºC in darkness. Then, the mycelium and conidia formed were scraped from the media surface with a spatula and suspended in autoclaved water. Spores were separated from the mycelium by sieving the content through four layers of fine sterile cheese-cloth. Finally, spore density was determined using a hemacytometer (Thomas Scientific, Philadelphia PA) and then adjusted to 10^6 spores g^{-1} substrate with autoclaved water.

4.2.2.2. *Glomus intraradices* 510

Glomus intraradices 510 (AMF) in expanded clay was kindly provided by Dr. H. von Alten, University of Hannover. For all experiment which included mycorrhiza, the inoculum was incorporated in expanded clay at a rate of 5% of total substrate volume.

4.2.2.3. *Rhizobium etli* G12

R. etli G12 (G12) was stored at -80 ºC in cryo vials. For production of the bacterium inoculum, pellets containing bacteria was transferred from cryo vials onto plates containing solid King's B medium for 36 h at 28 ºC (King et al., 1954). A loop of bacteria was transferred from the pre-culture into an Erlenmayer flask containing 100 ml of liquid King's B medium. The bacterium was cultured for 36 h at 28 ºC on a rotary shaker at 100 rpm. The bacterial suspension was centrifuge at 5000 g for 20 min at 10 ºC. The resulting pellets was re-suspended in sterile ¼ concentrated Ringer-solution (Merk) and the concentration was adjusted to an optical density of 2 at 560 nm ($OD_{560} = 2$).

4.2.2.4. *Aphis gossypii*

Aphis gossypii was maintained on susceptible cotton cv. Çukurva 1518, propagated in 300 g pots containing sandy loam soil in an incubator. The incubator was set to 25 ± 2ºC with a 16 h diurnal light. Apterous adult aphids were collected from heavily infected cotton leaves and immediately used for the bioassays.

4.2.3. Bioassays

4.2.3.1. Single and dual application of Fo162 with AMF or G12 on *A. gossypii* performance

To assess whether a co-application of Fo162 with AMF or G12 lead to additive or synergistic biocontrol activity towards *A. gossypii*, two bioassays were setup as follow:

Bioassay 1. Two week old squash plants were inoculated with water, Fo162, AMF or Fo162+AMF.

Bioassay 2. Two week old squash plants were inoculated with water, Fo162, G12 or Fo162+G12.

Fo162 was applied with 5 ml of a spore suspension at a concentration of 1×10^6 CFU g^{-1} of substrate dispensed over 3 holes around the selected plant base. AMF contained in expanded clay was incorporated at a rate of 5% of total substrate volume. G12 was applied with 5 ml pot^{-1} of a bacterial suspension (OD$_{560}$ = 2) as a drench around the stem base of the selected plant. Fo162 and G12 inoculations were repeated 1 week later as previously indicated by Dababat and Sikora (2007) and Reimann et al. (2008). Two weeks after the first inoculation, 10 wingless adult aphids were introduced, on the abaxial side of the third leaf of each plant and retained by a clip-cage. After 48 hours, the clip-cages were removed to avoid leaf damage. Flowers were continuously removed to avoid fruit development. Eight plants per treatment were used and the experiment was conducted twice. The plants were randomly arranged in a growth chamber at $25 \pm 3°C$ with 16 h day^{-1} diurnal light. Twenty days after aphid introduction, the number of aphids per plant was determined.

4.2.3.2. Spatially-separated dual inoculation of Fo162 and G12 on *A. gossypii* performance

Shoots of 2 week old squash plants were detached from the root system and then the basal part of the shoot split longitudinally into 2 sections over a length of 5 cm. Each section of the shoot was then placed in a separate 11 cm pot, filled with 300 g of autoclaved substrate (Chapter 2, Section 2.5). The two pots were separated approximately 1-cm from each other (Fig. 4.1). The split-root plants were maintained in a growth chamber for 2 weeks at $25 \pm 3°C$ with 16 h day^{-1} diurnal light to promote the development of adventitious roots.

The two sections of the split-root plants were considered as 'inducers' and the shoot was considered as 'responder' section. For a simultaneous but spatially separated inoculation with the biocontrol agents, one root section was inoculated with Fo162, whereas the other root section was inoculated with G12. Three controls were included in the experiment, in which both root sections were inoculated with Fo162, G12, or water (Fig. 4.1). The fungus was applied at a density of 1×10^6 CFU g^{-1} of soil dispensed in 3 holes around the selected inducer root section. At the same time, the bacterium was applied with 5 ml pot^{-1} of a bacterial suspension (OD$_{560}$ = 2) as a drench around the root section. This inoculation was

repeated 1 week later. Two weeks after the first inoculation, the responder sections were infected with 10 wingless adult aphids on the abaxial side of the third leaf, and retained by a clip-cage. After 48 hours, the clip-cages were removed to avoid leaf damage. Flowers were continuously removed to avoid fruit development. Six plants per treatment were used and the experiment was conducted twice. The plants were randomly arranged in a growth chamber under the conditions previously indicated. Twenty days after aphid introduction, the number of aphids per plant was determined.
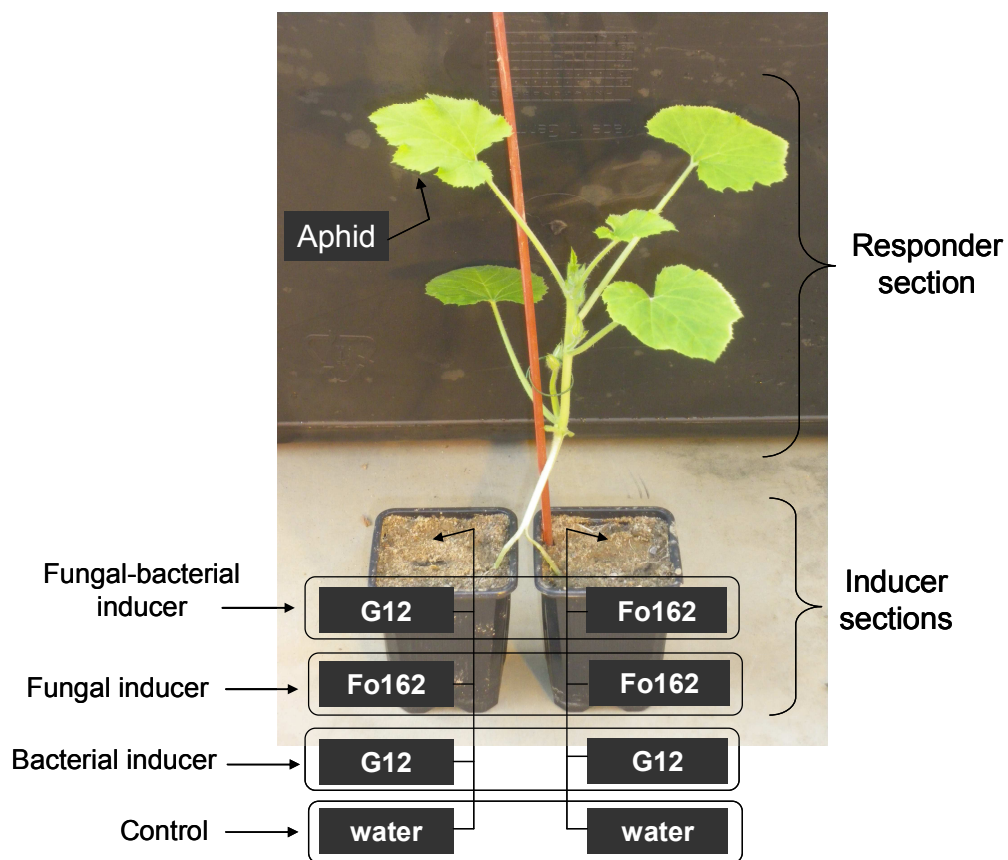
Figure 4.1. Split-root system to study systemic induced resistance with various combinations of spatially separated co-inoculation of *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* strain G12 towards *Aphis gossypii* on squash.

4.2.3.3. Influence of single application of Fo162 and G12 on *A. gossypii* host preference

Through a choice experiment, the effect of single inoculations with Fo162 or G12 on *A. gossypii* host preference was evaluated. For this, 2 week old squash plants were inoculated

with water (control), Fo162 or G12. The fungus and the bacterium were applied as previously described. These inoculations were repeated 1 week later. Two weeks after the first inoculation, the plants were organized in the following pairs: a) Fo162-Fo162, b) Fo162-G12, c) Fo162-control, d) G12-control, e) G12-G12 and f) control-control.

Each pair of treated squash plants were connected by a rectangular plastic box (15 x 8 x 4.5 cm), in which an opening (4.5 x 0.5 cm) had been cut out at each side. The petiole of the third leaf of one plant was passed through each hole (Fig. 4.2). The box was divided in two sections and labeled as A and B (Fig 4.2). Immediately thereafter, ten wingless aphid adults were placed at the border between section A and B, the boxes were then closed with a plastic lid (15 x 8 x 0.7 cm). Each lid contained three 3 cm ventilation holes, covered by a 70 μm mesh nylon sieve. The boxes were held in place by buret clamps attached to lab support stands (Fig. 4.2).

Each plant pair was repeated five times and the experiment was conducted twice. The plant pairs were randomly arranged in a growth chamber under the conditions indicated above. After 48 h, the distribution of the aphids over the two leaves was determined for each plant pair.
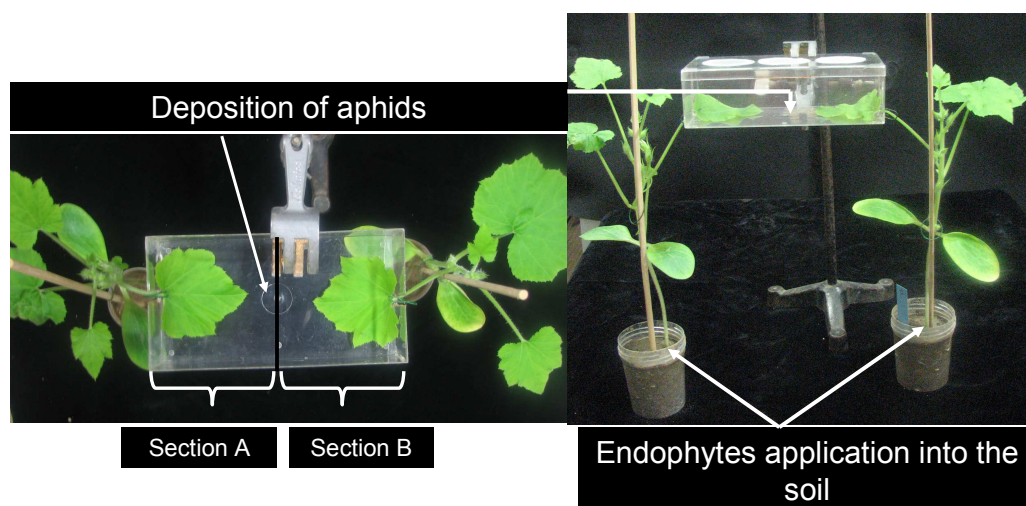

Figure 4.2. Design of a choice experiment with intact squash plants to study the effect of *Fusarium oxysporum* Fo162 and *Rhizobium etli* G12 treated plants on *Aphis gossypii* host preference.

4.3. Results

4.3.1. Single and dual application of Fo162 with AMF or G12 on *A. gossypii* performance

4.3.1.1. Bioassay 1, Fo162 and AMF

The ability of Fo162, AMF or a combination of the two endophytes to reduce *A. gossypii* population development was assessed in two pot experiments. Twenty days after the introduction of 10 aphids, the numbers of *A. gossypii* were significantly lower on plants threated with either Fo162 or AMF when compared to those on the control plants (Fig. 4.3; Exp.1: *P*=0.0139, LSD=117.96; Exp.2: *P*=<0.0001, LSD=30.681). However in Experiment 1, the reduction in number of aphids was not as distinct for AMF-treated plants in comparison to control plants (Fig. 4.3). The aphid numbers on the plants inoculated with a combination of Fo162 and AMF was not significantly different from those found on the individual endophyte treatments, Fo162 or AMF, indicating that no additive effect with respect to aphid control was present.

4.3.1.2. Bioassay 2, Fo162 and G12

Another set of pot experiments was used to evaluate the ability of Fo162, G12 or a combination of the two endophytes to reduce *A. gossypii* population development. Twenty days after the introduction of 10 aphids, the numbers of *A. gossypii* were significantly lower on plants with the application of either Fo162 or G12 when compared to those on the control plants (Fig. 4.4; Exp.1: *P*=<0.0001, LSD=12.878; Exp.2: *P*=0.0031, LSD=12.916). The reduction averaged 23 and 15 percent for Fo162 and G12 in Experiment 1, respectively; while in Experiment 2 the average reduction was 16 and 12 percent for Fo162 and G12, respectively (Fig. 4.4). The aphid numbers on the plants inoculated with a combination of Fo162 and G12 was not significantly different from those found on the individual endophyte treatments, Fo162 or G12, indicating that no additive effect with respect to aphid control had occurred.

Figure 4.3. Effect of individual and combined application of *Fusarium oxysporum* strain Fo162 (Fo162) and *Glomus intraradices* strain 510 (AMF) on *Aphis gossypii* performance in squash. Vertical bars represent the standard error of the mean. Columns with different letters are significantly different after LSD test ($P \leq 0.05$, n=8).
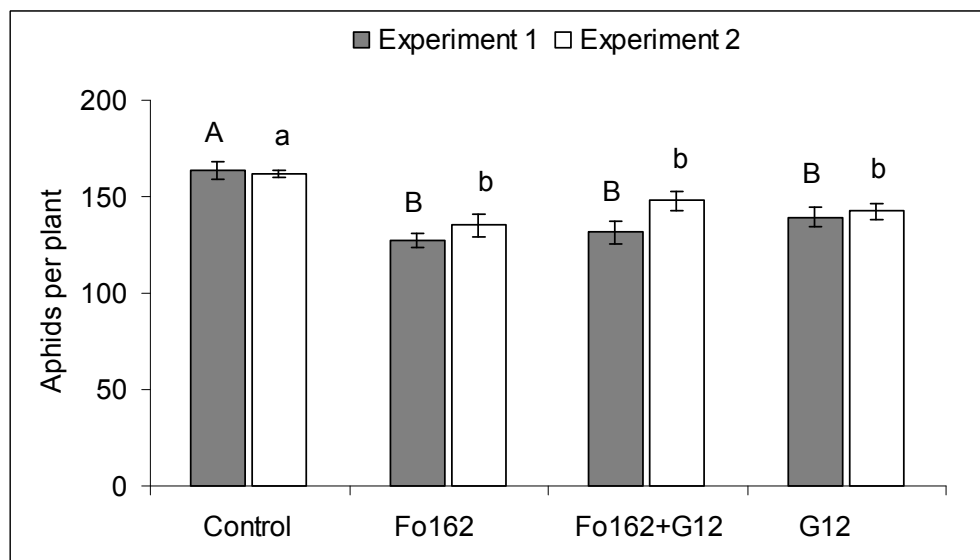
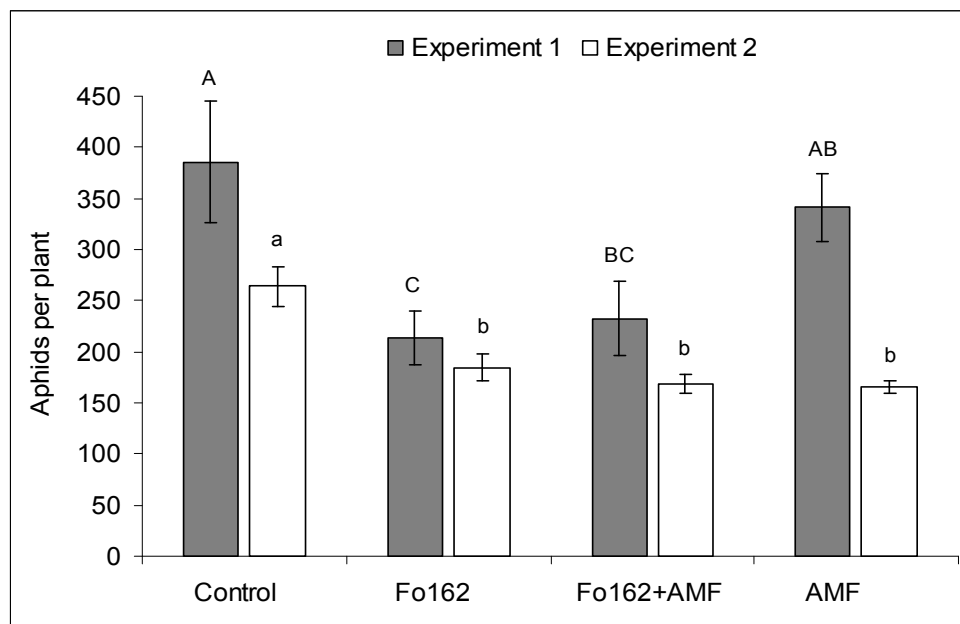

Figure 4.4. Effect of individual and combined application of *Fusarium oxysporum* strain Fo162 (Fo162) and *Rhizobium etli* strain G12 (G12) on *Aphis gossypii* performance in squash. Vertical bars represent the standard error of the mean. Columns with different letters are significantly different after LSD test ($P \leq 0.05$, n=8).

4.3.2. Spatially-separated dual inoculation of Fo162 and G12 on *A. gossypii* performance

A split-root experiment was used to monitor the ability of Fo162, G12 or a combination of the two endophytes to systemically reduce the *A. gossypii* population growth, when the biocontrol organisms were applied on the root system in a spatially separated way (Fig. 4.1). Twenty days after the introduction of 10 aphids per plant, the *A. gossypii* numbers on the leaves of the split-root plants inoculated with either Fo162 (Fungal inducer) or G12 (Bacterial inducer), were significantly lower than the aphid numbers on the control plants treated with water in two independent experiments (Fig. 4.5; Exp.1: *P*=0.0048, LSD=108.24; Exp.2: *P*=0.0037, LSD=37.464). When the plant roots were inoculated with Fo162 at one side and G12 at the other (Fungal-bacterial inducer) of the same plant, the aphid population reduction was similar to those plants treated with Fo162 or G12 at the inducer sections. The results demonstrated that the combined but spatial inoculation with the two microorganisms did not cause a synergistic or additive increase in the biocontrol efficacy.

Figure 4.5. Effect of individual and spatially separated combination of *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* strain G12 on *Aphis gossypii* performance in squash plants. Vertical bars represent the standard error of the mean. Columns with different letters are significantly different after LSD test (*P* ≤ 0.05, n=6).
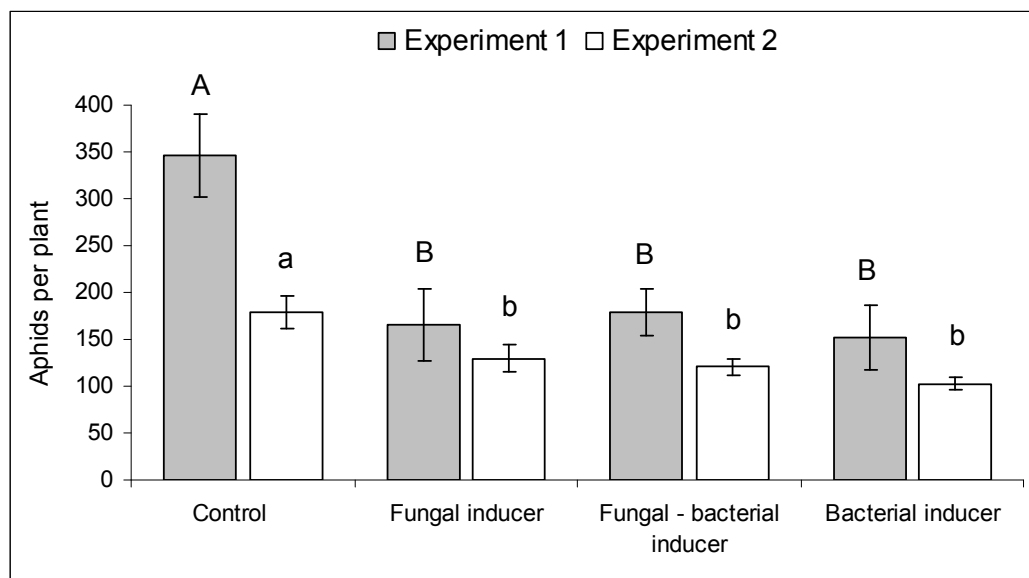
4.3.3. Influence of single application of Fo162 and G12 on *A. gossypii* host preference

Through a pair wise choice experiment, with squash plants either endophyte inoculated or non-treated, the leaf preference of *A. gossypii* was determined (Fig. 4.2). Forty-eight hours after introducing the aphids in the chamber in between the two plant leaves, the distribution of *A. gossypii* over the two leaves were not different when both plants had been inoculated with the same endophyte, Fo162 or G12, and when both plants were not inoculated (Control) (Table 4.1). When the leaf of an endophyte inoculated plant, Fo162 or G12, was paired with a non-inoculated plant, the distribution of aphids over the two leaves was not equal, with preference for the non-inoculated control leaf. When the leaves of plants inoculated with the different endophytes, Fo162 and G12, were paired, the majority of the aphids were found on the G12-inoculated plant leaf. All three unequal distributions were significant.

Table 4.1. Percentage of *Aphis gossypii* that migrated towards to one of the the two leaves of endophyte, Fo162 or G12, treated or untreated (Control) squash plants as determined by a pair wise choice experiment.

	Experiment 1			Experiment 2		
	Percentage of aphids present on leaf			Percentage of aphids present on leaf		
Treatment pair[a] (A-B)	A[b]	B[b]	*P*-value[c]	A	B	*P*-value
Control-Control	50 ± 4	40 ± 5	0.298	44 ± 4	48 ± 4	0.587
Fo162-Fo162	44 ± 6	40 ± 4	0.688	42 ± 6	46 ± 4	0.670
G12-G12	38 ± 8	38 ± 7	0.999	48 ± 6	42 ± 4	0.553
Fo162-Control	20 ± 5	46 ± 5	0.003	26 ± 5	64 ± 9	0.011
G12-Control	30 ± 6	46 ± 5	0.019	34 ± 5	54 ± 7	0.013
Fo162-G12	22 ± 6	46 ± 7	0.051	30 ± 4	58 ± 6	0.031

[a]The third leaf of two endophyte treated and/or untreated plants were placed on opposite sides of a closed plastic box, divided into two sections. Aphids were released in between the two leaves. [b]A and B refer to the plant treatment and the values represent the mean ± the standard error of five plants. [c]Difference in the proportions of aphids within each treatment pair were compared by a paired *t*-test at $P \leq 0.05$; n=5.

4.4. Discussion

The results from the current study confirmed the ability of both Fo162 and G12 to systemically influence *A. gossypii* performance on squash (Martinuz and Sikora, 2010; Menjivar, 2010). The simultaneous application of both biocontrol agents onto the squash root system resulted in a significant reduction in the *A. gossypii* final population. However, this reduction was not greater than the levels of population reduction obtained in treatments with the individual biocontrol agents. Furthermore, the simultaneous and spatially-separated application of Fo162 and G12 to the individual inducer sides of a split-root experiment also resulted in a significant decrease of *A. gossypii* final population, but this reduction again was not greater than the levels of control obtained when single applications of the endophytes were used. Thus, even when applied in a spatial way, the two biocontrol agents cannot improve the reduction in *A. gossypii* population in squash.

Martinuz et al. (2011) using a triple-split-root tomato plant setup demonstrated that spatially-separated but simultaneous inoculation of both Fo162 and G12 did not lead to additive reductions in the root-knot nematode (*Meloidogyne incognita)* infection, suggesting that similar defense mechanisms, triggered by Fo162 and G12, may be involved in induced resistance towards both *A. gossypii* and *M. incognita*. Rajendran et al. (2011) reported that following soil treatments of cotton plants with the endophytic bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* the population of *A. gossypii* was effectively reduced under greenhouse conditions. Similarly, Kempel et al. (2009) demonstrated that *G. intraradices* was able to induce resistance towards *Spodoptera littoralis* (Lep., Noctuidae) in four different grass plants. In an attempt to elucidate the bases of induced resistance against *A. gossypii*, Omer et al. (2001) sprayed cotton plants with jasmonic acid (JA). It was found that JA-induction reduced adult aphid survival 40% and reproduction 75%, when compared to the control. McConn et al. (1997) showed that jasmonate is essential for induction of resistance against insects. It was observed that *Bradysia impatiens* (Dip., Sciaridae) caused high mortality of an Arabidopsis mutant (fad3-2, fad7-2, fad8) that is deficient in the jasmonate precursor linolenic acid. However, application of exogenous methyl jasmonate substantially protected the mutant plants and reduced their mortality. Conversely, Inbar et al. (2001)

sprayed cotton plants with benzo (1,2,3) thiadiazole-7-carbothioic acid (S) methyl ester (BTH), an elicitor of systemic acquired resistance (SAR), concluding that SAR induction via the salicylic acid pathway in cotton has negligible effect on *Bemisia tabaci* (Hom., Aleyrodidae) and *Helicoverpa armigera* (Lep., Noctuidae).

A choice experiment with intact squash plants demonstrated that *A. gossypii* migrated to a lesser extent towards leaves of endophyte inoculated plants than to leaves of un-inoculated plants. Vicari et al. (2002) paired leaf disks taken from ryegrass plants treated with *N. lolii* and/or *G. mosseae* or from endophyte-free plants. They found that larvae of *Phlogophora meticulosa* (Lep., Noctuidae) preferred to feed on endophyte-free plants than on endophyte-inoculated plants regardless of mycorrhizal infection status. Nevertheless, a choice experiment with intact plants showed that *P. meticulosa* preferred to feed on endophyte-free plants than on *G. mosseae*-inoculated plants (Vicari et al., 2002). In this respect, Omer et al. (2001) showed that *A. gossypii* host preference was reduced by more than 60% on JA-induced leaves compared with controls determined by a choice experiment with leaf disks of cotton plants. This suggests that a JA-dependent signaling pathway is involved in the observed negative effect on aphid preference and performance.

An SA-dependent pathway has been proposed in endophytic *Fusarium* induced resistance towards pathogens and nematodes in studies with asparagus and tomato (He and Wolyn, 2005; Dababat and Sikora, 2007; Selim, 2010). Conversely, a rhizobacteria-mediated induced resistance was shown to be controlled by JA and ethylene (ET) pathways (Pieterse et al., 1998; Pieterse et al., 2001). Several studies on the cross-talk between the JA- and SA-dependent signaling pathways have shown that negative interactions can occur, with consequences for host resistance against pest and/or pathogens (Bostock et al., 2001). Thus, the lack of additive systemic effects against *A. gossypii* reported in this study could be related to antagonism of the JA- and SA-dependent signaling pathways; however this hypothesis needs to be evaluated.

4.5. Conclusions

The biological activity of single or combined application of the antagonists *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and *Rhizobium etli* strain G12 was investigated in this study. From the results the following can be concluded:

1. The individual application of *F. oxysporum*, *G. intraradices* or *R. etli* results in a significant reduction in the population of *A. gossypii* on squash leaves.

2. Concomitant enhancement with *F. oxysporum* together with *G. intraradices* or with *R. etli* does not lead to significant synergistic interactions.

3. The simultaneous application of *F. oxysporum* and *R. etli* on a split-root experiment showed that the simultaneous but spatially-separated inoculation of both endophytes did not lead to significant additive effects with respect to reducing *A. gossypii* population.

4. The leaf preference of *A. gossypii* in a pair-wise choice experiment on squash plants showed that *A. gossypii* preferred to feed on endophyte-free plants than on the endophyte-inoculated plants. When plants inoculated with Fo162 and G12 were paired, the majority of *A. gossypii* was found on G12-inoculated plants.

5. Overall, the present research results seems to indicate that a more detailed characterization of the defense mechanisms, triggered by the various endophytic biocontrol agents together with knowledge on the sensitivity of pest insects to specific defense mechanisms would help to increase efficacy when microorganisms are combined to improve biocontrol of pathogens and pests

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Chapter 5

Interactions between the endophytic fungus *Fusarium oxysporum* strain Fo162 and the endophytic bacterium *Rhizobium etli* strain G12

5.1. Introduction

The facultative fungus*, Fusarium oxysporum,* is a common and highly variable soil inhabitant. This species of *Fusarium* includes a number of important plant pathogens that affects negatively crops of economic importance worldwide. Nevertheless, most *F. oxysporum* isolates are saprophytes that feed on the organic matter in the soil. Others have been isolated from the internal tissue of plant roots after surface disinfection. Non-pathogenic *Fusarium* isolates are important to crop production, since some have been shown to induce resistance in host plants; thus, increasing the plant's ability to defend itself from pathogen and pest attack (Alabouvette et al., 1998; Larkin and Fravel, 1999; Pereira et al., 1999; Trouvelot et al., 2002; Sikora et al., 2008).

There are many bacteria associated with the root system having different forms of influence on the plant. In the recent past, rhiozobacteria that live on the surface of the root and that stimulate plant growth (plant growth promoting rhizobacteria, PGPRs) were of great interest to biological science (Kloepper and Schroth, 1981; Liu et al., 1995; Hallmann et al., 1998). More recently, endophytic bacteria that have been isolated from internal plant tissues after surface-sterilization of the root surface have become interesting to those working in plant health management (Vidal et al., 1998; Hasky-Günther et al., 1998; Sikora et al., 2007). They have been shown to be able to colonize the root internal but also the shoots leaves and flowers (Hallman et al., 2001). The potential these bacteria have in pest and disease management was demonstrated in several studies (Sturz et al., 2000; El-Batanony, 2007; Sikora et al., 2007). The authors showed that inoculation of different vegetables with rhizobacteria strains reduced diseases caused by soil-born pathogens and nematodes, thus resulting in promotion of plant growth and health. This led to the term plant health promoting rhizobacteria PHPR (Sikora et al., 2007).

The inconsistency in the level of biocontrol, often associated with application of microorganisms to plants, has been considered a limiting factor when using antagonistic bacteria for plant protection against soil-born pests and pathogens. The lack of consistency is probable due to the fact that all management strategies are based on the application of a single microorganism usually for economic reasons. In such cases, control is limited to a short period of time and is futher influenced by existing fluctuations in biotic and abiotic conditions in the soil (Dunne et al., 1998; Reimann et al., 2008). Consequently, it has been suggested that co-inoculation strategies or combining different microorganisms to enhance biocontrol activity is needed (Dunne et al., 1998; El-Tarabily et al., 2000; Chaves et al., 2009; Sikora et al., 2010).

The use of different types of beneficials that colonize the plant differently or at different times and beneficials with different mechanisms of action could be more effective in pest and disease suppression. For instance, inhibition of pathogens by antibiosis; the production of microbial inhibiting metabolites like siderophore; competition for nutrients; competitive exclusion due to initial site colonization; induction of plant resistance; degradation or inhibition of hatch; or germination and production of plant growth enhancement through phytohormones production that increases tolerance (Deshwal et al., 2003; El-Mehalawy, 2004; Sikora et al., 2007).

However, direct and indirect interactions that could have negative impact on co-inoculated endophytes themselves needs to be taken into account before such strategies are fully developed. For example, concomitant application of *Trichoderma harzianum* and arbuscular mycorrhizal fungi (AMF) inhibited the development and colonization of AMF (Wyss et al., 1992), whereas AMF establishment was enhanced in presence of another isolate of the same fungus (Filion et al., 1999). Other studies demonstrated that combined inoculation of *Glomus intraradices* and *Rhizobium etli* G12 for the biocontrol of *M. incognita* led to additive effects (Reimann et al., 2008). Similar tests with co-application of *F. oxysporum* and *Bacillus firmus* also lead to additive reduction in the number of *Radopholus similis* that penetrated banana roots and not the synergistic activity that was expected (Mendoza and Sikora, 2009). When the endophyte *F. oxysporum* and the egg pathogen *Paecilomyces lilacinus* were combined the

result was not even additive with regards to control of *R. similis* in banana (Mendoza and Sikora, 2009). In recent studies the combination of *F. oxysporum* and *R. etli* G12 produced similar levels of control as single inoculations towards *M. incognita* and *Aphis gossypii* in tomato and squash respectively (Martinuz and Sikora, 2010).

These results seems to indicate that competition between the antagonistic microorganisms, both in or on the root system could lead to competition and thereby reduced colonization and/or activity of one or more of the microorganisms being used to enhance biological control efficacy through co-inoculation.

The objectives of the present investigations were to:

1. Evaluate the *in vitro* interactions between the endophytic fungus *Fusarium oxysporum* strain Fo162 and the endophytic bacterium *Rhizobium etli* strain G12.

2. Determine the *in vivo* direct interactions between the endophytic fungus *Fusarium oxysporum* strain Fo162 and the endophytic bacterium *Rhizobium etli* strain G12.

3. Assess the influence of spatial separation of the endophytes *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* strain G12 on endophyte colonization.

5.2. Materials and methods

5.2.1. Plants and substrate

Tomato plants cv. Moneymaker were used in all experiments. Plants were cultivated until ready for experiments as described in Chapter 2, Sections 2.4.1 and 2.4.2. The substrate for all experiments in this study was prepared as indicated in Chapter 2, Section 2.5.

5.2.2. Microorganisms

5.2.2.1. *Fusarium oxysporum* Fo162

F. oxysporum Fo162 (Fo162) was stored at -80 °C using cryo vials (Cryobank^{TM}, Master Group, Merseyside, UK). For production of the fungal inoculum for all experiments, a single frozen pellet was transferred onto Potato Dextrose Agar (PDA) plates (Difco, Sparks, MD, USA) supplemented with 150 mg 1^{-1} of streptomycin sulfate and chloramphenicol to avoid bacterial contamination. The fungal culture was incubated for 3 weeks at 25 ºC in darkness. Then, the mycelium and conidia formed were scraped from the media surface with a spatula and suspended in autoclaved water. Spores were separated from the mycelium by sieving the content through four layers of fine sterile cheese-cloth. Finally, spore density was determined using a hemacytometer (Thomas Scientific, Philadelphia PA) and then adjusted to 10^6 spores g^{-1} substrate with autoclaved water.

5.2.2.2. *Rhizobium etli* G12

R. etli G12 (G12) was stored at -80 ºC in cryo vials. For production of the bacterium inoculum, pellets containing bacteria was transferred from cryo vials onto plates containing solid King's B medium for 36 h at 28 ºC (King et al., 1954). A loop of bacteria was transferred from the pre-culture into an Erlenmayer flask containing 100 ml of liquid King's B medium. The bacterium was cultured for 36 h at 28 ºC on a rotary shaker at 100 rpm. The bacterial suspension was centrifuge at 5000 g for 20 min at 10 ºC. The resulting pellets was

re-suspended in sterile ¼ concentrated Ringer-solution (Merk) and the concentration was adjusted to an optical density of 2 at 560 nm ($OD_{560} = 2$).

5.2.3. *In vitro* interactions between Fo162 and G12

5.2.3.1. Culture media

A dual culture system using 9 mm diameter Petri dishes filled with different nutrient media was used to assess interactions of G12 on Fo162 development. Each dish was filled with 25 ml of either potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), tryptone soya agar (TSA), or root tissue agar (RTA). PDA, SDA, and TSA were prepared according to fabricant instructions. For RTA 30 g of fresh root tissue from 4 week old tomato plants was macerated and mixed with 500 ml of distilled water containing 18 g of agar. The mixture was then calibrated to 1 l and autoclaved at 121 °C for 20 min.

The bioassay was performed separately on each of the media. G12 was initially cultured on solid King's B, (Chapter 2, Section 2.1.3) and was then streaked in two parallel lines on PDA, SDA, TSA, or RTA in the dishes. Each line was placed 2 cm from the center of the Petri dish. Control plates were not inoculated with the bacterium (Fig. 5.1). The plates were then incubated for 3 days at 28 ºC in the dark. Thereafter, a 5 mm plug of Fo162, taken from 3 weeks old culture grown on PDA (Chapter 2, Section 2.1.1), was plated in the center of each Petri dish (Fig. 5.1). The plates were then incubated for 3 to 5 days at 28 ºC in the dark. Each treatment was replicated 10 times and the bioassay was repeated. The influence of the bacterium on fungal growth was assessed by measuring radial mycelia growth.

5.2.3.2. Duration of bacterial incubation

The amount of antimicrobial compounds produced by bacteria in culture is influenced by the duration of fermentation or growth on culture media. To test this factor a dual culture system was used to test the influence of the G12 incubation time on Fo162 radial growth on 9 mm diameter Petri dishes containing 25 ml of PDA. G12 cultured on solid King's B, was

streaked in two parallel lines on PDA. Each line was placed 2 cm from the center of the Petri dish. Control plates were not inoculated with the bacterium. The plates were then incubated for 1,2,3,4 and 5 days at 28 ºC in the dark (Fig. 5.2). Thereafter, a 5 mm plug of Fo162, taken from a 3 week old culture grown on PDA was plated in the center of each Petri dish. The plates were then incubated for 3 days at 28 ºC in the dark (Fig. 5.2). Each treatment was replicated 6 times and the bioassay was repeated once. The influence of the bacterium on fungus growth was evaluated by measuring radial mycelia growth after 3 days exposure.
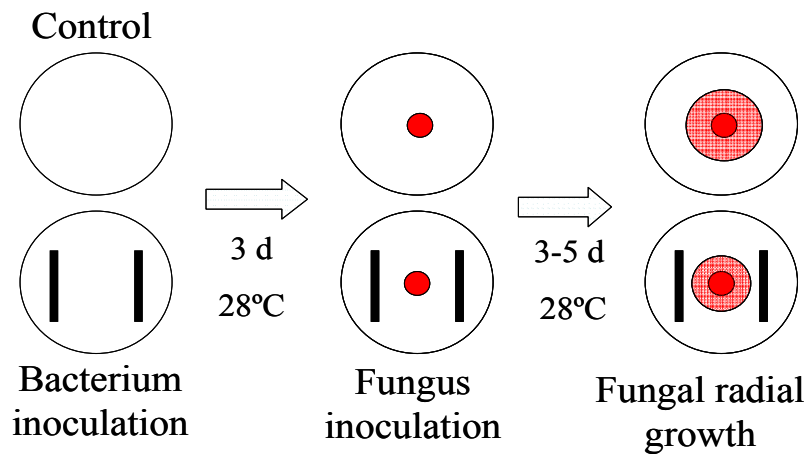

Figure 5.1. Flow diagram of the bioassay used for assessment of the influence of *Rhizobium etli* G12 on the growth of *Fusarium oxysporum* Fo162. Modified from Reimann (2005).
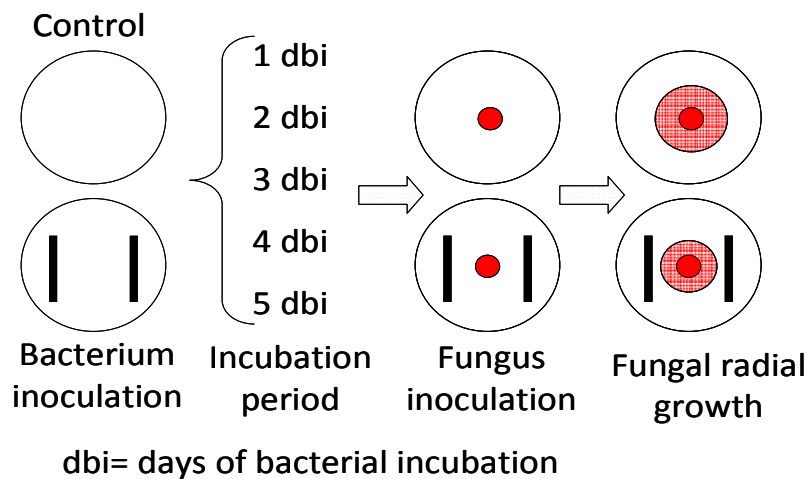

Figure 5.2. Flow diagram of bioassay used to test the influence of *Rhizobium etli* G12 incubation time on *Fusarium oxysporum* Fo162 radial growth. Modified from Reimann (2005).

5.2.4. *In vivo* interaction between Fo162 and G12

5.2.4.1. Single antagonist inoculations

To study the *in vivo* interactions between Fo162 and G12 a pot experiment was performed in which the plants were inoculated singly or in combination. Tomato plants, cultivated as indicated above, were inoculated with Fo162, G12 or Fo162+G12. Plants treated with water serve as controls. Fo162 was applied with 5 ml of a spore suspension at a concentration of $1x10^6$ CFU g^{-1} of substrate dispensed over 3 holes around the selected plant base. G12 was applied with 5 ml pot^{-1} of a bacterial suspension (OD_{560} = 2) as a drench around the stem base of the selected plant. The same inoculation procedure was repeated two weeks after transplanting to improve root colonization. Pots were arranged in a completely randomized design and kept in a greenhouse at 25 ± 3°C with 16 h day^{-1} supplemental artificial light. Each treatment was replicated 6 times and the experiment was repeated.

The existence of an interaction between the two endopyhtes was evaluated by determining the efficiency of Fo162 root colonization. The density of G12 was not determined due to the similarity of the colonies with other root inhabiting microorganisms. Root colonization by Fo162 was determined 4 weeks after the first inoculation by the surface sterilization and imprint method, followed by plating on PDA as described in Chapter 3 Section 3.2.3.2.

5.2.4.2. Split-root systemic activity experiment

The systemically mediated influence of G12 on Fo162 colonization was tested with a split-root system (Fig. 5.3). Tomato plants were transplanted into plastic pots containing 300 g of an autoclaved substrate and kept under green house condition at 27 ± 3°C with 16 h day^{-1} supplemental artificial light. Two weeks after transplanting, plants were separated from the root system 0.5 cm above the soil surface and the shoot split in half longitudinally 5 cm. The lower leaves were pruned to reduce transpiration. Two plastic pots were filled with 300 g of the autoclaved substrate and placed one next to one another (Fig. 5.3). Each half of the split shoot was inserted into the substrate of a pot. The plants were kept in a greenhouse for 2

weeks under the same condition previously indicated. During this period, the shoots developed a strong adventitious root system and were suitable for further experimentation.

Each side of the split-root system was then labeled as either the inducer root or the responder root (Fig. 5.3). The inducer sides were inoculated with Fo162, G12 or water. One week later the responder sides were inoculated with Fo162. The split-root plants were arranged in a completely randomized design in a greenhouse under the same condition previously indicated. Each treatment was replicated six times and the experiment was repeated. Four weeks after inducer treatment, the roots on the responder side were washed free of substrate with tap water. Root colonization by Fo162 on the responder half was then determined trough the surface sterilization and imprint method, followed by plating on PDA as detailed in Chapter 3 Section 3.2.3.2.
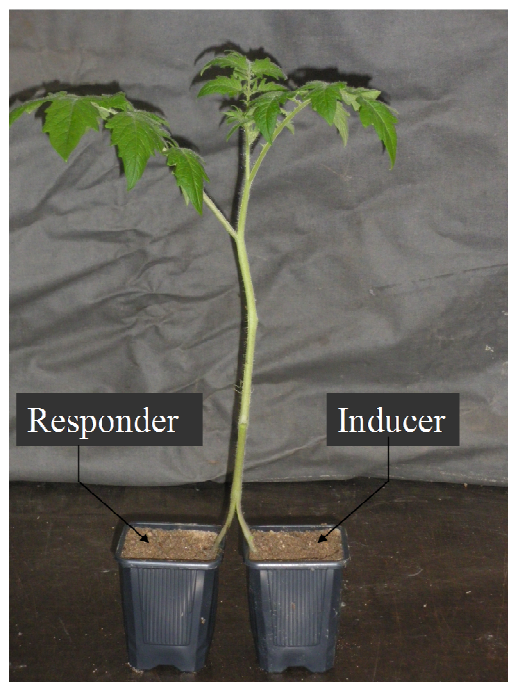

Figure 5.3. Split-root plant system used to determine the systemic interaction of *Rhizobium etli* G12 and *Fusarium oxysporum* Fo162 on tomato.

5.3. Results

5.3.1. *In vitro* interactions between Fo162 and G12

5.3.1.1. Culture media

In the dual culture bioassays, Fo162 radial growth was significantly inhibited when growing in the presence of G12 when compared to the control plates (Fig. 5.4 a and b). The results were similar in all media tested, except in SDA which showed no difference between treatments. Observations performed under light microscope showed no morphological changes in Fo162 mycelium induced by the presence of G12.

5.3.1.2. Duration of bacterial incubation

Fo162 radial growth decreased significantly as bacterial incubation time increased. Non-lineal regression analysis demonstrated a negative relationship (Fig. 5.5). Fungal radial growth decreased in the first experiment: 10, 17, 22, 26 and 28 percent and in the second experiment: 22, 35, 43, 48 and 50 percent in comparison to the control, when the bacterium was cultured for 1, 2, 3, 4 or 5 days before inoculating Fo162, respectively.
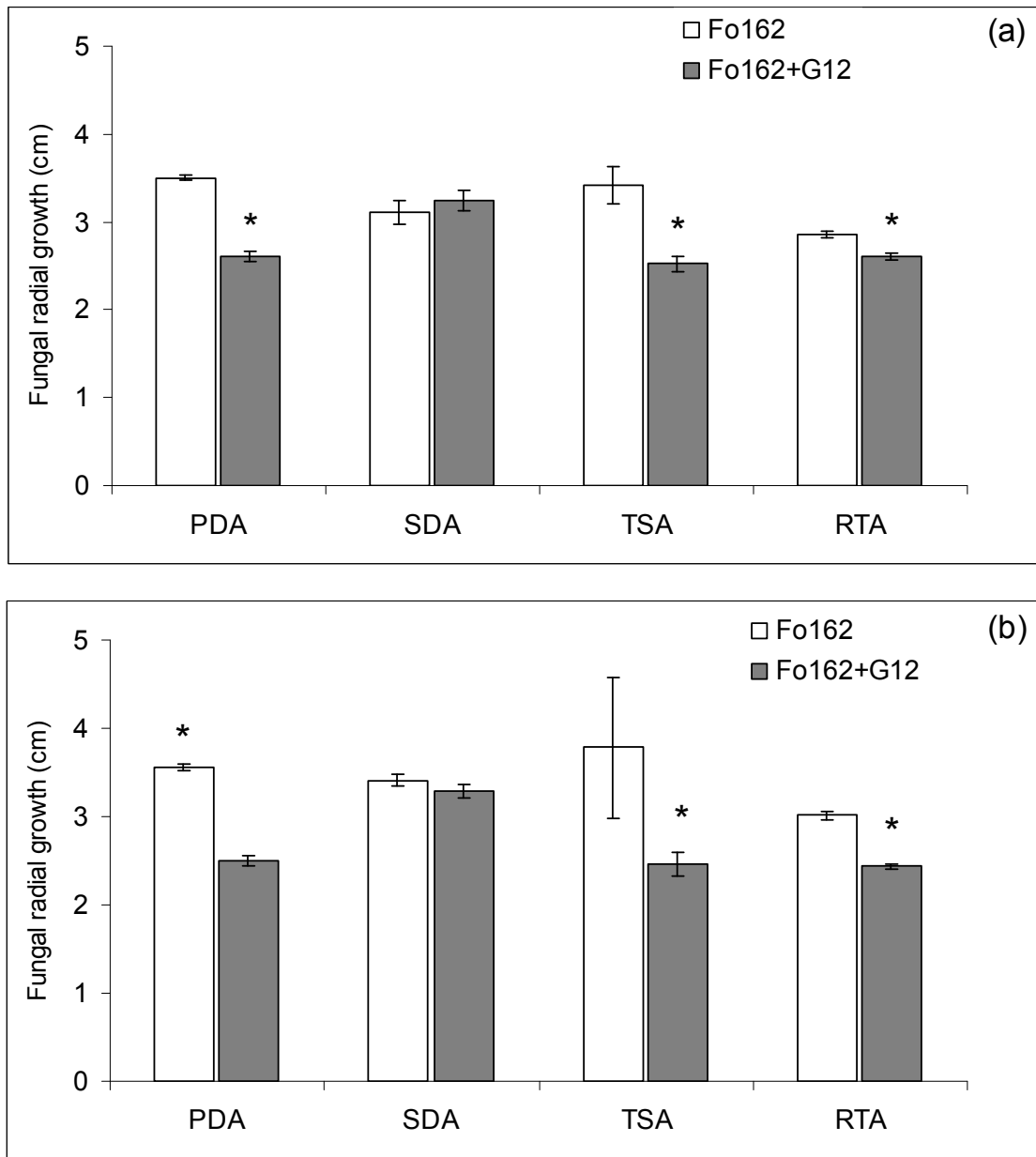
Figure 5.4. Influence of *Rhizobium etli* strain G12 (G12) on *Fusarium oxysporum* strain Fo162 (Fo162) radial growth *in vitro* after a period of 3 to 5 days of co-culture at 28 ºC. Vertical bars represent standard error of the mean values. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=10). (a) Experiment 1 and (b) Experiment 2. PDA, potato dextrose agar; SDA, Sabouraud dextrose agar; TSA, tryptone soya agar; RTA, root tissue agar.

(a)

Rg(t) = 2.36 + 1.24exp(-0.34t)

$R^2 = 0.97$
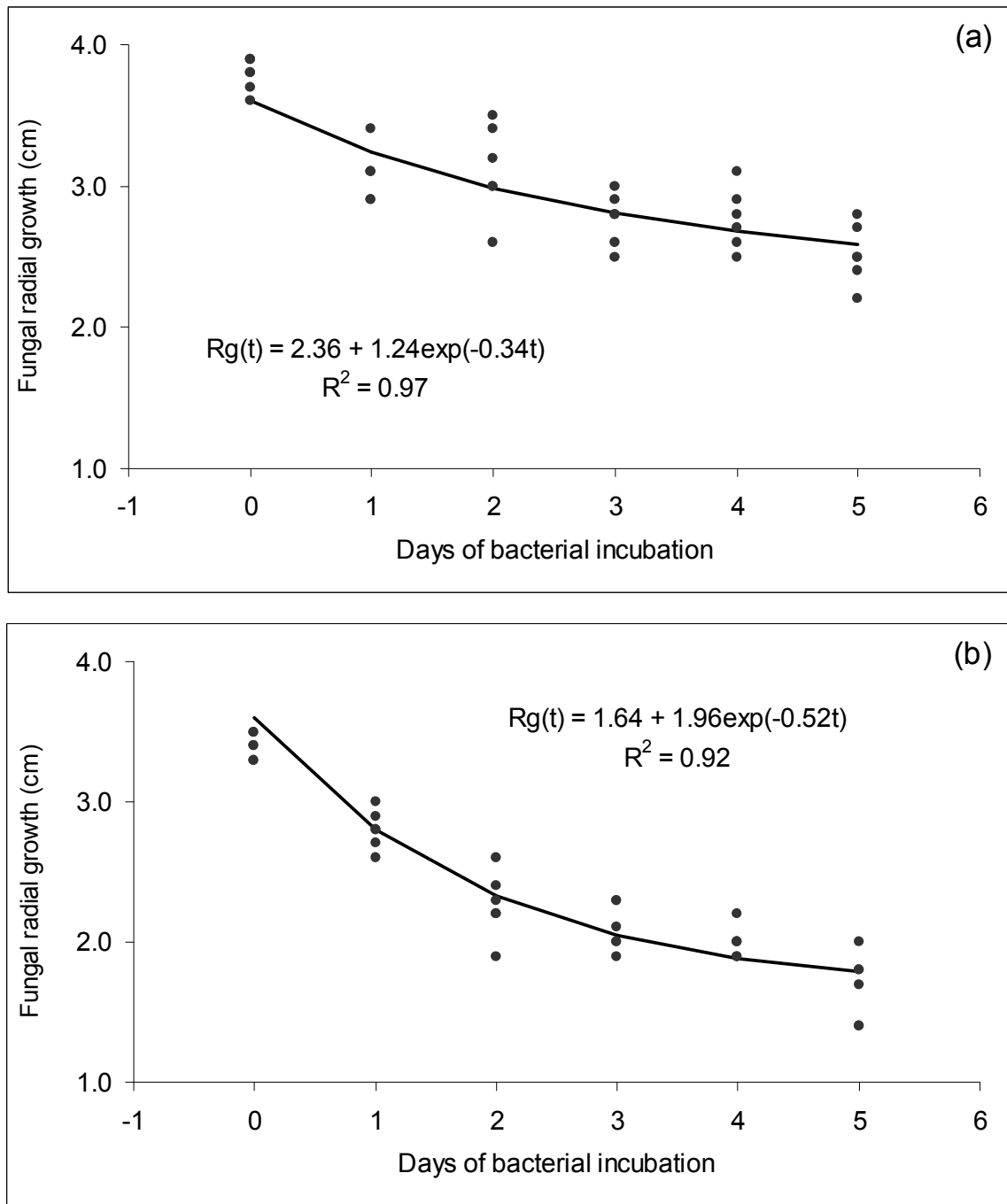
(b)

Rg(t) = 1.64 + 1.96exp(-0.52t)

$R^2 = 0.92$

Figure 5.5. Influence of *Rhizobium etli* G12 on *Fusarium oxysporum* Fo162 radial growth assessed under *in vitro* conditions and its non-lineal regression curve [Rg(t) = α + (3.6-α)exp(-β*t); $P \leq$ 0.05, n=6]. (a) Experiment 1 and (b) Experiment 2.

5.3.2. *In vivo* interactions between Fo162 and G12

5.3.2.1. Single antagonist inoculations

Four weeks after the first inoculation of the two antagonists, the colonization of Fo162 in the roots co-inoculated with the two organisms was significantly reduced 34 and 49 percent in the two experiments, when compared to the fungus colonization on plants treated only with Fo162, respectively. The fungus Fo162 could not be detected in the treatment with water nor in the treatment with the bacteria G12.

5.3.2.2. Split-root systemic activity experiment

Four weeks after Fo162 inoculation on the responder side of the split-root system, systemic inhibition of Fo162 root colonization was observed. In the presence of G12 at the inducer side, the percentage colonization by Fo162 on the responder side was repressed approximately 50 percent when compared to the water controls in the two experiments (Fig. 5.7). Inoculation of the inducer section of the root system with water or with Fo162 had no significant effect on Fo162 colonization on the responder side. In this case, the level of Fo162 colonization on the responder side was 17.8 and 22.2 percent in Experiment 1 and 2, respectively, which was similar to the levels of colonization obtained with water as the inducer, being 15.6 and 25.0 percent in both experiments, respectively (Fig. 5.7).
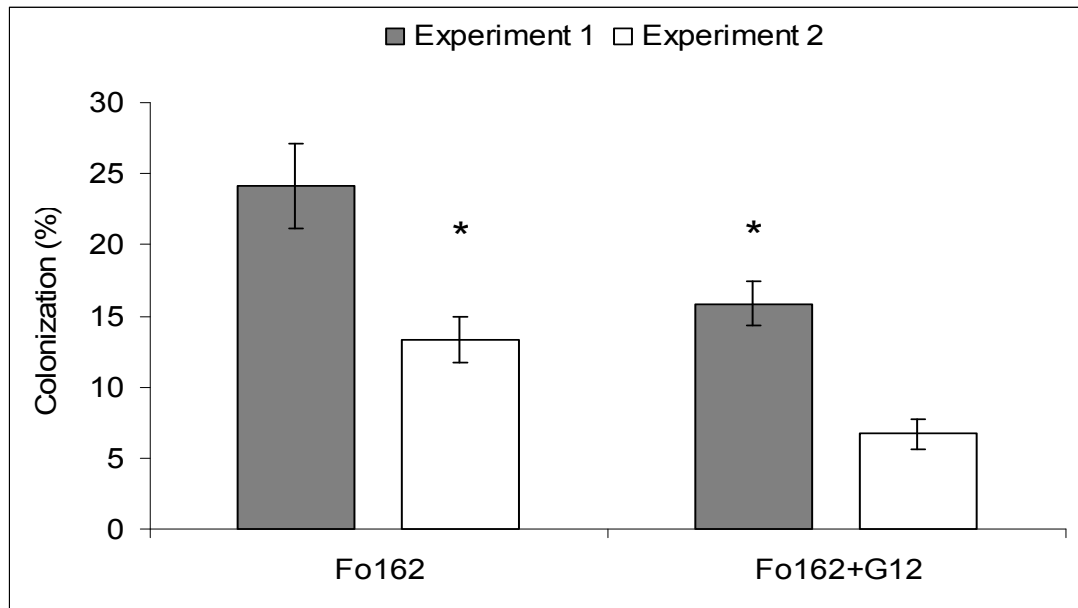
Figure 5.6. *Fusarium oxysporum* strain Fo162 (Fo162) endophytic colonization of tomato roots in the presence of *Rhizobium etli* G12 (G12) four weeks after the first inoculation. Vertical bars represent standard error of the mean values. Means with (*) indicates significantly different after *t*-test for independent samples ($P \leq 0.05$, n=6).
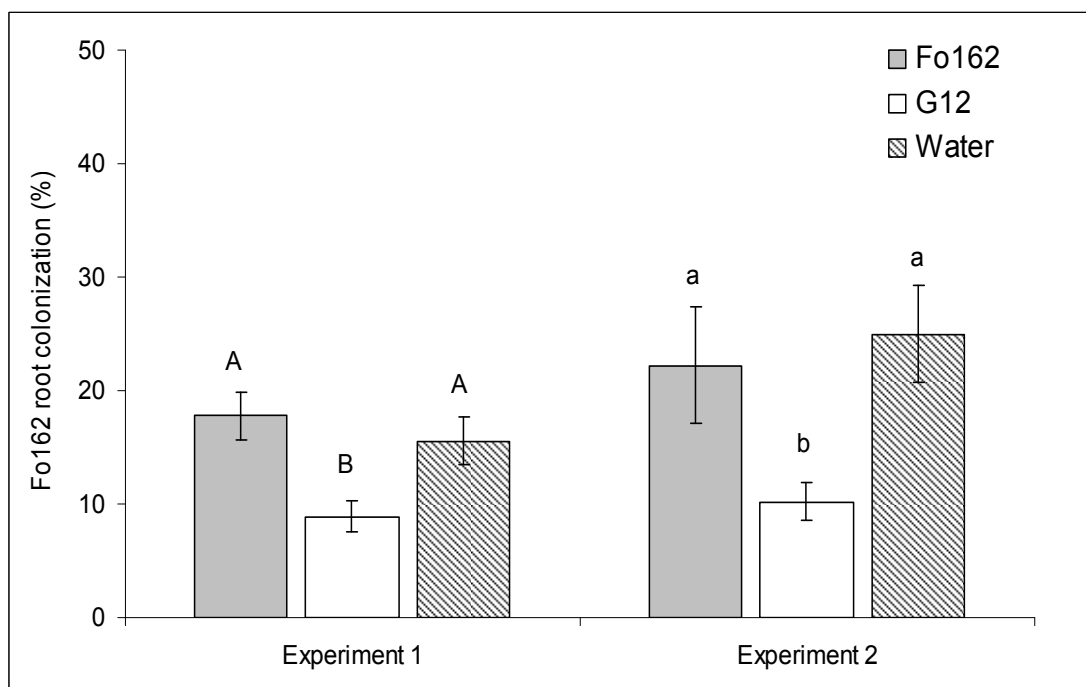
Figure 5.7. Systemic activity of *Rhizobium etli* strain G12 (G12) on *Fusarium oxysporum* strain Fo162 (Fo162) colonization in the responder half of a split-root system. Vertical bars represent standard error of the mean values. Columns with different letters are significantly different after LSD test ($P \leq 0.05$, n = 6).

5.4. Discussion

The results obtained in Chapter 3 and 4 of this thesis research showed that single and dual application of Fo162 and G12 enhanced plant resistance to *M. incognita* in the root system and *Aphis gossypii* on the shoots. However, the combined application of both antagonistic endophytes did not result in additive or synergistic levels of activity towards the nematode or the insect. There was evidence that indicate that a concomitant application of two antagonists, each having different modes of action and colonization nitchs in the root, could lead to improved suppressive efficacy towards nematodes (Reimann et al. 2008; Chaves et al. 2009). Conversely, it also has been reported that non-additive effects against nematodes can occur when using combination of beneficial bacteria and fungi (Castillo et al., 2006; Mendoza and Sikora, 2009). Although the role of specific endophytic microorganisms, especially fungi and bacteria, in the suppression of insect and nematode infection has been thoroughly reviewed (Clay, 1989; Breen, 1994; Saikkonen et al., 1998; Azevedo et al., 2000; Sikora et al., 2007; Sikora et al., 2008), little is known about biological enhancement of pests following concomitant inoculation of different types of endophytic microorganisms (Gaylord et al., 1996; Diedhiou et al., 2003; Martinuz and Sikora, 2010). The *in vitro* and *in vivo* interaction studies conducted here between Fo162 and G12 were devised to investigate concomitant interactions between the two organisms on tomato.

The antibiosis tests performed in this investigation resulted in a reduction in Fo162 radial growth when challenged with G12. This reduction also was positively correlated with increased duration of G12 incubation on solid media. Similar results were reported by El-Botanony et al. (2007) who tested, *in vitro* and *in vivo*, the inhibitory effect of cultural filtrates of wild *Rhizobium* spp. against soil-born pathogens including *F. oxysporum*. They found that the inhibitory effects, expressed as an inhibition of mycelial growth increased with increasing concentration of the filtrates. They concluded that the endophytes tested exerted antagonistic activity towards soil-born pathogenic fungi suppressing their growth and increasing plant resistance.

In the present studies, the inhibitory effects observed between G12 and Fo162 could be related, in part, to antagonism caused by the accumulation of toxic metabolites produced by the endophytic bacterium while reproducing on root exudates. The lack of an additive or synergistic increase in biological control, detected in these studies, therefore could be related to the fact that both microorganisms are applied at the same time and this causes direct and immediate competion for space and nutrients. To test this last hypothesis, an experiment was performed to study the influence of G12 on the colonization of Fo162. Results showed lower root colonization by Fo162 when it was applied in association with G12.

The manner in which these organisms colonize the host plant may be important in the interaction. Olivian et al. (2003) studied the colonization of flax roots by the non-pathogenic *F. oxysporum* strain Fo47. They reported that the fungus actively colonized the root surface. Afterwards, the fungus penetrated into epidermal cells, and then colonized heavily the hypodermis. On the other hand, Hallmann et al. (2001) studied the external and internal colonization of potato and Arabidopsis roots by G12 containing a plasmidborne *trp* promoter green fluorescent protein transcriptional fusion. They found that the bacterium colonized the entire root surface; however, they preferentially colonized root tips and the emerging lateral roots. Internal colonization was located in epidermal cells, as well as in or near vascular tissues. Furthermore, Diedhiou et al. (2003) co-inoculated *G. coronatum* and Fo162 in an attempt to improved *M. incognita* biocontrol. They showed that the presence of Fo162 stimulated *G. coronatum* colonization and that roots already colonized by *G. coronatum* were not internally colonized by Fo162. The manner in which they interact therefore may be influenced by nitch factors. Very quick colonization of the surface of the root by G12 may reduce the ability of Fo162 to recognize the host plant or may even repell colonization due to the toxic metabolites that were shown here to have inhibitory activity on Fo162.

Systemic induced resistance (SIR) has been reported as a mode of action of G12 (Hasky-Günther et al., 1998). SIR can have activity towards fungi as has been shown by Mwangi et al. (2002). Therefore, it can not be excluded as a factor affecting the interaction with non-pathogenic *Fusarium*. Thus, a split-root experiment was designed to analyze the systemic influence caused by G12 on the colonization of Fo162. The results showed again a

significant decrease in Fo162 colonization, when the inducer half of the split-root system was inoculated with the bacterium.

These results are similar to those of Liu et al. (1995) who used a split-root system to study the SIR produced by *P. putida* and *S. marcescens* towards Fusarium wilt caused by *F. oxysporum* f. sp. *cucumerinum*. They concluded that both bacteria were able to delayed disease symptom development and retarded colonization of the pathogen. Nevertheless, they could not explain whether the SIR activity produced by the bacterial strains was related to transportable substances induced or produced by the bacteria. Conversely, Reitz et al. (2000) using split-root essay demonstrated that lipopolysaccharides of G12 works as inducing agent of systemic resistance towards *Globodera pallida* in potato. Futhermore, van Peer and Schippers (1992) associated lipopolysaccharides of plant growth promoting *Pseudomonas* spp. to SIR against Fusarium wilt caused by *F. oxysporum* f. sp. *dianthi* in carnation. Mwangi et al. (2002) demonstrated that *Bacillus sphaericus* B43 induce systemic resistance to the fungal wilt pathogen *F. oxysporum* f.sp. *lycopersici* in tomato. They found that peroxidase activity in the stem of bacterial treated plants significantly increased over a short period of time; while the β-1,3-glucanase activity was not affected by the prencense of the bactirium.

The overall results of these studies suggest that G12 affected not only *M. incognita* and *A. gossypii*, but also had a negative effect on Fo162 establishment in the root system. Therefore, synergistic levels of biological control towards the nematode or the insect, due to dual application of both antagonists, did not materialize as seen in Chapters 3 and 4. Three modes of action have been considered important in nematode control with endophytic bacteria (Hallmann et al., 2001; Sikora et al., 2007): a) preemptive colonization, b) direct antagonism though toxic metabolites and c) systemic induced resistance. Conversely, in insect biological control (a) displayed phenotypic plasticity (e.g. increase of trichome densities), (b) allelochemical(s) production and (c) systemic induced resistance are considered important modes of action when using endophytes as biological agents (Breen, 1994; Traw and Bergelson, 2003).

Although these modes of action have been demonstrated to have an influence on nematodes and insects in the present studies, they have negative impact to the extent that they also can lead to the inhibition of Fo162 development and therefore the lack of additive or synergistic effect towards *M. incognita* and *A. gossypii* as shown in Chapters 3 and 4. Therefore, it is important to highlight that, although both Fo162 and G12 are recognized as beneficial microorganisms, their mutual direct or indirect interactions on each other also have to be taken into account in co-inoculation strategies in biocontrol.

5.5. Conclusions

The *in vitro* and *in vivo* interactions between *F. oxysporum* Fo162 and *R. etli* G12 were investigated in the present study. From the results obtained the following can be concluded:

1. The *in vitro* dual-culture challenge tests showed that the bacterium caused a significant reduction in *F. oxysporum* radial growth. The reduction in radial growth was positively correlated with the bacterial incubation period.

2. In greenhouse experiments the simultaneous application of *F. oxysporum* and *R. etli* resulted in a reduction of *F. oxysporum* colonization, indicating that the fungus can be directly repressed by the bacterium.

3. A split-root experiment showed that the simultaneous but spatially-separated inoculation of both *F. oxysporum* and *R. etli* resulted in a reduction in *F. oxysporum* colonization, indicating that the fungus can be systemically repressed by the bacterium.

4. The results demonstrate the need to test for incompatibility between microbial biocontrol agents when considering co-inoculation treatment.

5.6. References

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Chapter 6

Interactions between the endophytic fungus *Fusarium oxysporum* strain Fo162 and the root-knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*

6.1. Introduction

The root-knot nematode, *Meloidogyne incognita*, is an obligate biotrophic plant parasite that evolved unique strategies to infect a large number of plants. The infective second-stage juveniles (J2) penetrate behind the root tip and migrate between cells to invade the vascular cylinder. Each J2 then establishes and maintains a permanent group of feeding cells, known as giant cells, which constitute the exclusive source of nutrients for the developing nematode. Hyperplasia and hypertrophy of the surrounding cells lead to the formation of the typical root gall, constituting the primary visible symptom of infection (Caillaud et al., 2008). The galls interfere with normal root function, reducing water and nutrient uptake; consequently, leading to stunting, wilting and growth inhibition (Sikora and Fernandez, 2005). Root-knot nematodes are managed by means of different strategies, such as cultural, biological and chemical measures. These measures of control include crop rotation, resistant varieties, flooding, fallow, incorporation of organic matter, soil solarization, steam heating, nematicides and biological control (Noling and Becker, 1994; Manzanilla-Lopez, 2004; Sikora et al., 2005).

The mutualistic endophytic fungus *Fusarium oxysporum* strain Fo162 (Fo162) is considered as a potential non-chemical alternative (Hallman et al., 2001; Sikora et al., 2007). However, variations in the level of biocontrol mediated by the endophyte constitute a disadvantage for commercial application. This lack of consistency may be caused by different factors such as the level of root colonization by the endophyte and fluctuations in biotic and abiotic conditions in the soil (Dunne et al., 1998). Thus, a more detailed characterization of the defense mechanisms triggered by Fo162 together with knowledge on the sensitivity of *M. incognita* to specific defense mechanisms would help in increasing efficacy when endophytes are used to enhance biocontrol of pathogens and pests.

In order to further study plant defense responses towards *M. incognita* triggered by the endophyte Fo162, the interaction between the endophyte and the model plant *Arabidopsis thaliana* was assessed. *A. thaliana* is a flowering plant belonging to the Brassicaceae that is parasitized by *M. incognita* and some species of *Fusarium* (Sijmons et al., 1991; van Hemelrijck et al., 2006; Hallmann et al., 2001). In the 1980s, *Pseudomonas syringae* became the first pathogen demonstrated to be able to infect Arabidopsis and to cause disease symptoms in laboratory experiments (Katagiri et al., 2002), establishing the relevance of this plant as a scientific tool for plant-microbe interaction studies. Since then, Arabidopsis has been used to unravel basic plant defense response mechanisms that reach beyond the plant biology boundaries (Micali et al., 2008). The progress made on Arabidopsis pathology research has recently gained importance; particularly, the study of obligate biotrophic and hemibiotrophic pathogens has contributed to the understanding of the molecular basis of basal and isolate-specific defense mechanisms (Micali et al., 2008).

The objectives of these investigations were to:

1. Determine the ability of *F. oxysporum* Fo162 to colonize the root system of *A. thaliana* wild type Columbia.

2. Evaluate the influence of Fo162 on plant growth parameters.

3. Determine the effect of single inoculation with Fo162 on the penetration of *M. incognita*.

4. Evaluate the influence of single inoculation with Fo162 in a spatially-separated plant bioassay on root gall formation of *M. incognita*.

5. Investigate the effect of inducing host plant systemic resistance using different combinations of chemical elicitors on root gall formation of *M. incognita*.

6.2. Materials and methods

6.2.1. Plants and substrate

Arabidopsis plants of the wild type Columbia were used in all experiments. Plants were cultivated until ready for experiments as described in Chapter 2, Sections 2.4.1 and 2.4.4. The substrate for all experiments in this study was prepared as indicated in Chapter 2, Sections 2.5 and 2.6.6.

6.2.2. Microorganisms

6.2.2.1. *Fusarium oxysporum* Fo162

Fo162 was stored at -80 °C using cryo vials (Cryobank[TM], Master Group, Merseyside, UK). For production of the fungal inoculum for all experiments, a single frozen pellet was transferred onto PDA dishes (Chapter 2, Section 2.6.1). The fungal culture was incubated for 3 weeks at 25 ºC in darkness. Then, the mycelium and conidia formed were scraped from the media surface with a spatula and re-suspended in autoclaved water. Spores were separated from the mycelium by sieving the content through four layers of fine sterile cheese-cloth. Finally, spore density was determined using a hemacytometer (Thomas Scientific, Philadelphia PA) and then adjusted to 10^6 spores g^{-1} substrate with autoclaved water.

6.2.2.2. *Meloidogyne incognita*

M. incognita was maintained in a box (150 x 80 x 40 cm) filled with sandy loam and permanently cultivated with the susceptible tomato cv Furore in a greenhouse at 27 ± 5 °C. Nematode eggs were extracted from 2 month old heavily galled tomato roots, using the modified extraction technique of Hussey and Barker (1973). Roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. NaOCl was added to obtain a final concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was

then thoroughly washed with tap water through a sieve combination 250, 100, 45 and 25 μm mesh to remove the NaOCl. Eggs were collected on the 25 μm mesh sieve and then transferred to a glass bottle. The egg suspension was supplied with oxygen from an aquarium pump over 10 days to induce juvenile hatching. To separate active J2 from unhatched eggs or dead J2, a modified Baermann technique over 24 h was used (Oostenbrink, 1960). The collected active J2 were adjusted to 1000 J2 ml^{-1} and used immediately as inoculum.

6.2.3. Root colonization by Fo162

Two week old Arabidopsis plants placed in 4 cm diameter pots containing 50 g of a mixture of autoclaved sand:soil (3:1, v/v), were inoculated with Fo162 or with water (control). The fungus was applied as a spore suspension in 3 ml pot^{-1} at a concentration of 1 x 10^6 CFU g^{-1} of substrate. The inoculum was dispensed into three holes around the selected plants. For the water control, 5 ml sterilized water was used. Pots were arranged in a completely randomized design in a climatic chamber at 25 ± 3 °C with 16 h day^{-1} supplemental artificial light. Each treatment was replicated 10 times and the experiment was repeated.

Root colonization by Fo162 was determined 4 weeks after inoculation by washing the roots free of substrate with tap water. Thereafter, roots were surface sterilized by submersion in a 0.5% NaOCl solution for 3 min, followed by three rinses in sterile distilled water. The roots were then imprinted on PDA (Chapter 2, Section 2.6.1) to verify surface sterilization (Schulz et al., 1999). The surface sterilized roots were then cut into 0.5 cm sections and 18 root segments per treatment were randomly selected and placed on two Petri dishes containing PDA medium. After 4 to 7 days of growth at 25°C in the dark, fungi emerging from each root segment were phenotypically verified as being Fo162. These data were used for calculating the percentage of root colonization per root system, i.e. (the number of root segments colonized by Fo162/total number of root segments)*100 (Mendoza and Sikora, 2009).

6.2.4. Influence of Fo162 on plant growth promotion

6.2.4.1. Plant weight

Sterile seeds of Arabidopsis were sown in Petri dishes containing 25 g of a mixture of autoclaved sand:soil (3:1, v/v) and 7 ml of sterile water . The dishes containing 5 seeds dish^{-1} were incubated for two weeks in a climatic chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light (Fig. 6.1). Afterwards, each Petri dish was inoculated with Fo162 or with water (control). The fungus was applied as a spore suspension in 1 ml dish^{-1} at a concentration of 1 x 10^6 ml^{-1}. The inoculum was applied as a drench at the center of the Petri dish. For the water control, 1-ml sterilized water was used. Dishes were arranged in a completely randomized design in a climatic chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light. Each treatment was replicated 10 times and the experiment was repeated. Two weeks after inoculation, the 5 plants dish^{-1} were carefully uprooted and washed free of substrate with tap water. The plans were then blotted between two paper tissues and total fresh weight of the 5 plants dish^{-1} was recorded. This data were used to calculate plant fresh weight (shoot + root).

Figure 6.1. System used to study the influence of *Fusarium oxysporum* Fo162 on the plant fresh weight of *Arabidopsis thaliana*.

6.2.4.2. Root length and average diameter

Sterile seeds of *A. thaliana* wild type Columbia were sown in Petri dishes on 2% Knop medium (Chapter 2). The dishes containing 5 seeds plate^{-1} were incubated in a climatic chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light (Fig. 6.2). Two weeks later, each Petri dish was inoculated with Fo162 or with water (control). The fungal endophtye was applied as a spore suspension in 10 μl dish^{-1} at a concentration of 1 x 10^6 ml^{-1}. The inoculum was carefully spread across the center of the Petri dish. For the water control, 10 μl dish^{-1} of sterilized water was used. Dishes were arranged in a completely randomized design in a climatic chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light. Each treatment was replicated 10 times and the experiment was repeated. Two weeks after inoculation, plants shoots were carefully detached from their roots and root length and average diameter were measured with a Comair root length scanner (Hawker de Havilland) (Diedhiou et al., 2003).
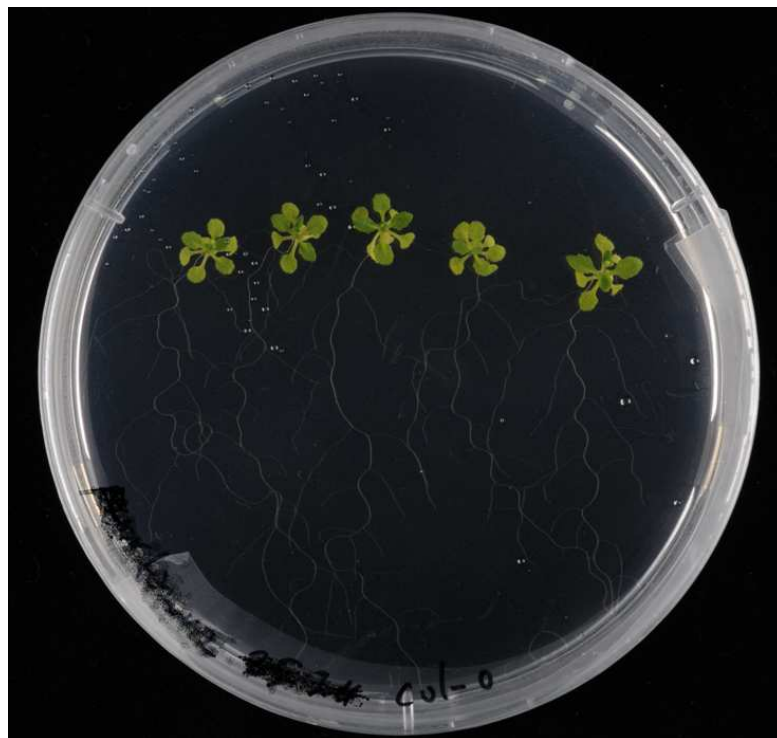

Figure 6.2. System used to study the influence of *Fusarium oxysporum* Fo162 on *Arabidopsis thaliana* wild type Columbia root length and average diameter.

6.2.5. Influence of Fo162 on *M. incognita* penetration

Two week old Arabidopsis plants placed in 4 cm diameter pots containing 50 g of a mixture of autoclaved sand:soil (3:1, v/v), were inoculated with Fo162 or with water (control). The inoculum was applied as detailed in Section 6.2.3. Pots were arranged in a completely randomized design in a climatic chamber at 25 ± 3 °C with 16 h day^{-1} supplemental artificial light. Each treatment was replicated 10 times and the experiment was repeated. Two weeks after fungal inoculation, each plant was inoculated with a 200 µl suspension containing 200 *M. incognita* J2. The nematode suspension was dispensed into three 0.5-cm-deep holes around the plants. Two weeks after nematode inoculation, nematode penetration was determined by staining the roots with 0.1% acid fuchsin solution, followed by heating to boiling using a microwave for 1.5 to 2 min (Ferris, 1985; Dababat et al., 2007). After cooling for 60 min, excess acid fuchsin was removed by rinsing the roots with tap water. The roots were then macerated twice for 10 s in 15 ml water at high speed (11000 rpm) using an Ultra Turrax® T25 (Whatman GmbH, Dassel, Germany). The macerated root suspension was adjusted to 50 ml with tap water and thoroughly mixed by shaking. From this, two winding-track counting trays (Hooper et al., 2005) were immediately filled with 10 ml aliquots each. The number of penetrated J2 was then counted under a stereomicroscope (100x magnification) and the total number of J2 per root system calculated.

6.2.6. Influence of spatial separated inoculation of Fo162 on *M. incognita* gall formation

Sterile seeds of *A. thaliana* wild type Columbia were sown in Petri dishes on 2% Knop medium. The dishes containing 10 seeds plate^{-1} were incubated in a climatic chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light. Two weeks later, each plant was transferred to a 3-section Petri dish containing 15 g of a mixture of autoclaved sand:soil (3:1, v/v) and 5 ml of sterile water in two of the sections (Fig. 6.3). One week after transplanting, the inducer side of the two roots was inoculated with Fo162. The roots at the responder side remained untreated. Plants treated with tap water at the inducer side served as controls. The fungus was applied as a spore suspension in 1-ml plate^{-1} at a concentration of 1 x 10^6 CFU ml^{-1}. The inoculum was dispensed in the center of the inducer section. For the water control, 1-ml

sterilized water was used. Two weeks after fungal inoculation, the responder root section of each plant was inoculated with a 200 μl suspension containing 200 *M. incognita* J2. The nematode suspension was placed at the center of the responder root section. Treatments were replicated eight times and the experiment was conducted twice. The plants were arranged in a completely randomized design in a growth chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light. Twenty days after nematode inoculation, the responder roots were collected and carefully washed free from substrate. The number of galls per root system was then determined by counting.
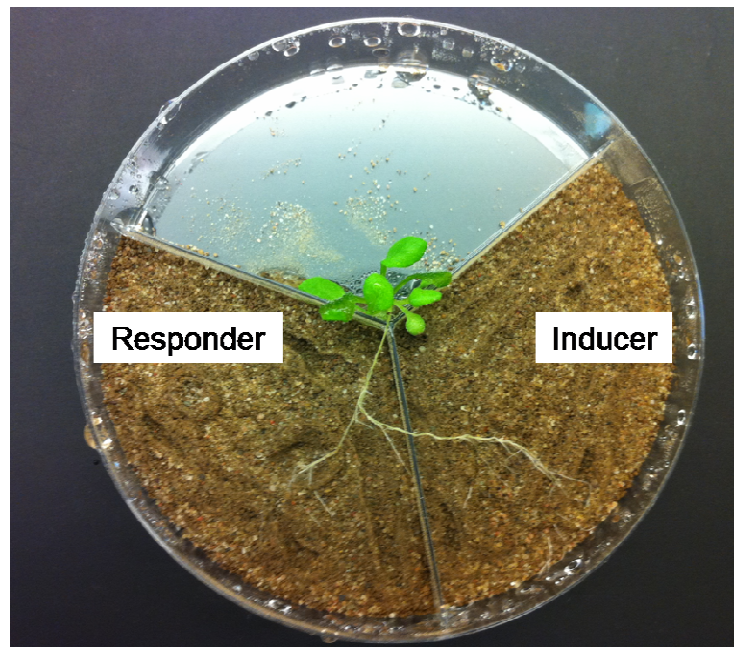

Figure 6.3. Split-root system used to study systemically induced resistance of *Fusarium oxysporum* strain Fo162 towards *Meloidogyne incognita* on Arabidopsis.

6.2.7. Induction of host plant systemic resistance by different combinations of chemical elicitors on *M. incognita* gall formation

The ability of two known chemical elicitors to induce systemic resistance in Arabidopsis towards *M. incognita* was investigated using salicylic acid (SA) and methyl jasmonate (MeJA). The experiment was setup as outlined in Section 6.2.6 and Fig. 6.3, with the following modification: the inducer compartment of the split-root system was treated with 5

ml of a mixture of different combinations of SA and MeJA, while the responder section received 5 ml of sterile water. Stock solutions of SA and MeJA substrates were prepared with concentrations of 20 mM and 10 mM respectively by adding the SA or MeJA 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 (Selim, 2010).

The following mixtures of chemical elicitors were used as treatments (data represent µM of SA + µM of MeJA):

0+0	0+10	0+50	0+100	0+250
10+0	10+10	10+50	10+100	10+250
50+0	50+10	50+50	50+100	50+250
100+0	100+10	100+50	100+100	100+250
250+0	250+10	250+50	250+100	250+250

One week after treating the inducer sides with the elicitors, the roots at the responder side were inoculated with a 200 µl suspension containing 200 *M. incognita* J2. Treatments were replicated four times and the experiment was conducted twice. The plants were arranged in a completely randomized design in a growth chamber at 25 ± 1 °C with 16 h day^{-1} supplemental artificial light. Twenty days after nematode inoculation, the responder roots were collected and carefully washed free from substrate. The number of galls per root system was then counted.

6.3. Results

6.3.1. Root colonization by Fo162 and influence on growth promotion

Fo162 was successfully re-isolated from Arabidopsis roots four weeks after fungal inoculation in two independent experiments (Fig. 6.4). In general, the level of the endophyte colonization was similar in both experiments, being 32.17 percent for Experiment 1 and 28.83 percent for Experiment 2. It was not possible to re-isolate the fungus from control plants. Arabidopsis root colonization by Fo162 resulted in a significant increase in plant fresh weight, root length and average root diameter in both experiments, when compared to endophyte-free plants (Table 6.1 and Fig. 6.5).

6.3.2. Influence of Fo162 on *M. incognita* penetration

Two weeks after nematode inoculation, *M. incognita* penetration was reduced significantly following Fo162 inoculation in Experiment 1 (P=0.0036) and Experiment 2 (P=0.0025), when compared to the nematode penetration of control plants treated with water (Fig. 6.6). Nematode penetration was reduced 50 percent in Experiment 1 and 35 percent in Experiment 2 when compared to the untreated control respectively.

6.3.3. Influence of spatial separated inoculation of Fo162 on *M. incognita* gall formation

A split-root experiment was used to monitor the ability of Fo162 to reduce systemically *M. incognita* gall development when both organisms were separately inoculated (Fig. 6.3). Twenty days after nematode inoculation, *M. incognita* gall development in the responder section of the split-root system was reduced significantly following fungal inoculation in Experiment 1 (P=0.010) and Experiment 2 (P=0.0011), when compared to the nematode gall development of control plants treated with water (Fig. 6.7).
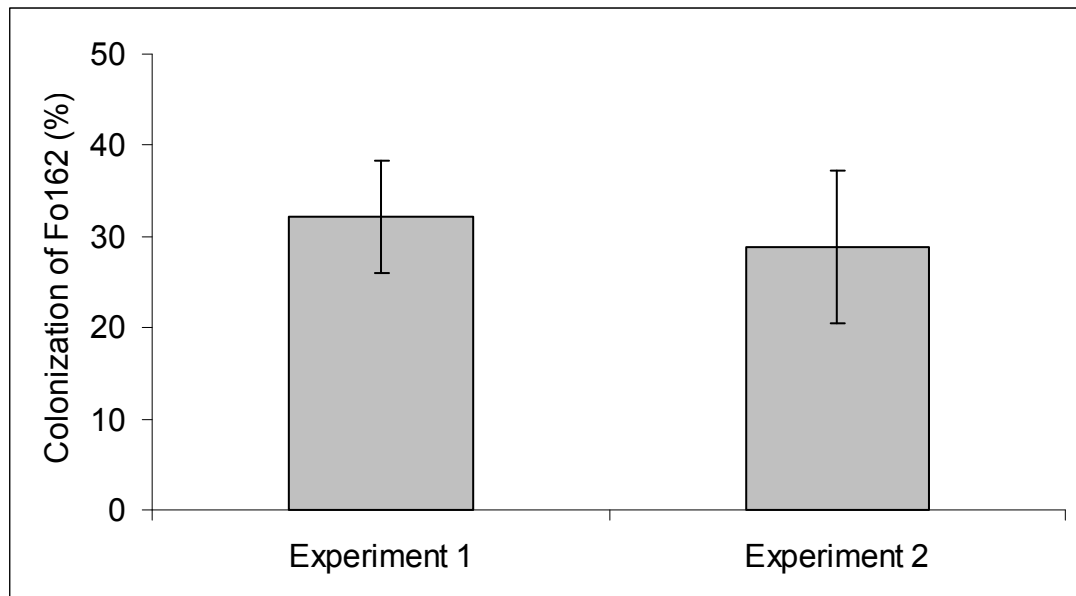
Figure 6.4. Re-isolation of *Fusarium oxysporum* strain Fo162 from the root system of Arabidopsis wild type Columbia, determined four weeks after fungal inoculation. Vertical bars represent standard error of the mean values (n=10).

Table 6.1. Effect of *Fusarium oxysporum* Fo162 on plant weight, root length and root average diameter of Arabidopsis wild type Columbia.

Treatment	Experiment 1			Experiment 2		
	Plant fresh weight (mg)	Root length (cm)	Root average diameter (μm)	Plant fresh weight (mg)	Root length (cm)	Root average diameter (μm)
Control	15.41 ± 1.55[a]	13.63 ± 1.76	25.12 ± 0.56	17.42 ± 2.56	24.20 ± 5.18	23.68 ± 0.83
Fo162	24.76 ± 2.61	52.69 ± 4.10	29.54 ± 0.65	25.28 ± 2.79	39.06 ± 3.32	30.33 ± 1.34
P-value[b]	0.0065*	<0.0001*	0.0001*	0.0554	0.0301*	0.0008*

[a]Data represent mean ± standard error. [b]Data with (*) indicates significant differences with the *t*-test for independent samples ($P \leq 0.05$, n=10).
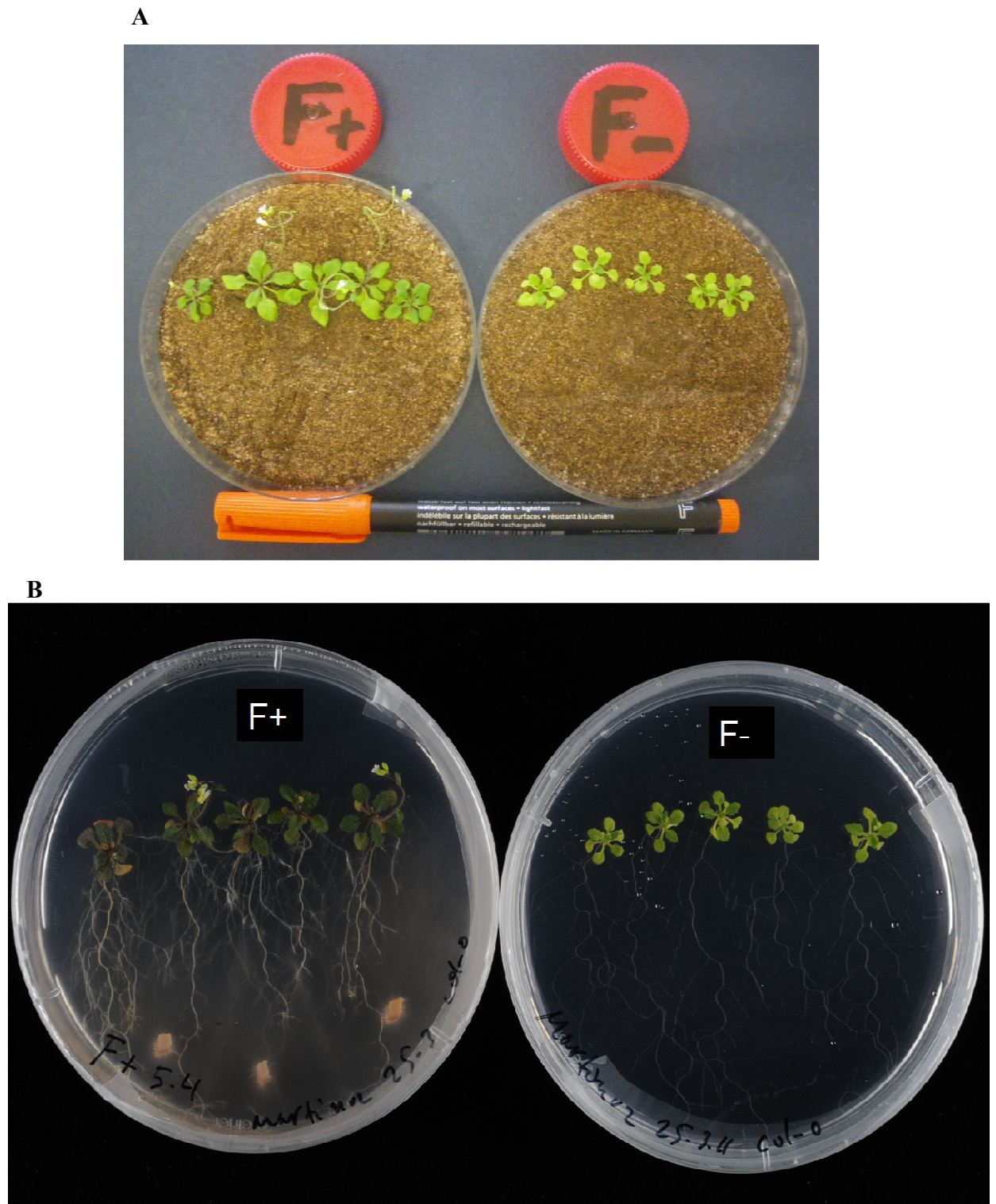
A

B

Figure 6.5. Selected treatment to illustrate the effect of *Fusarium oxysporum* Fo162 on Arabidopsis shoot (A) and root system (B).
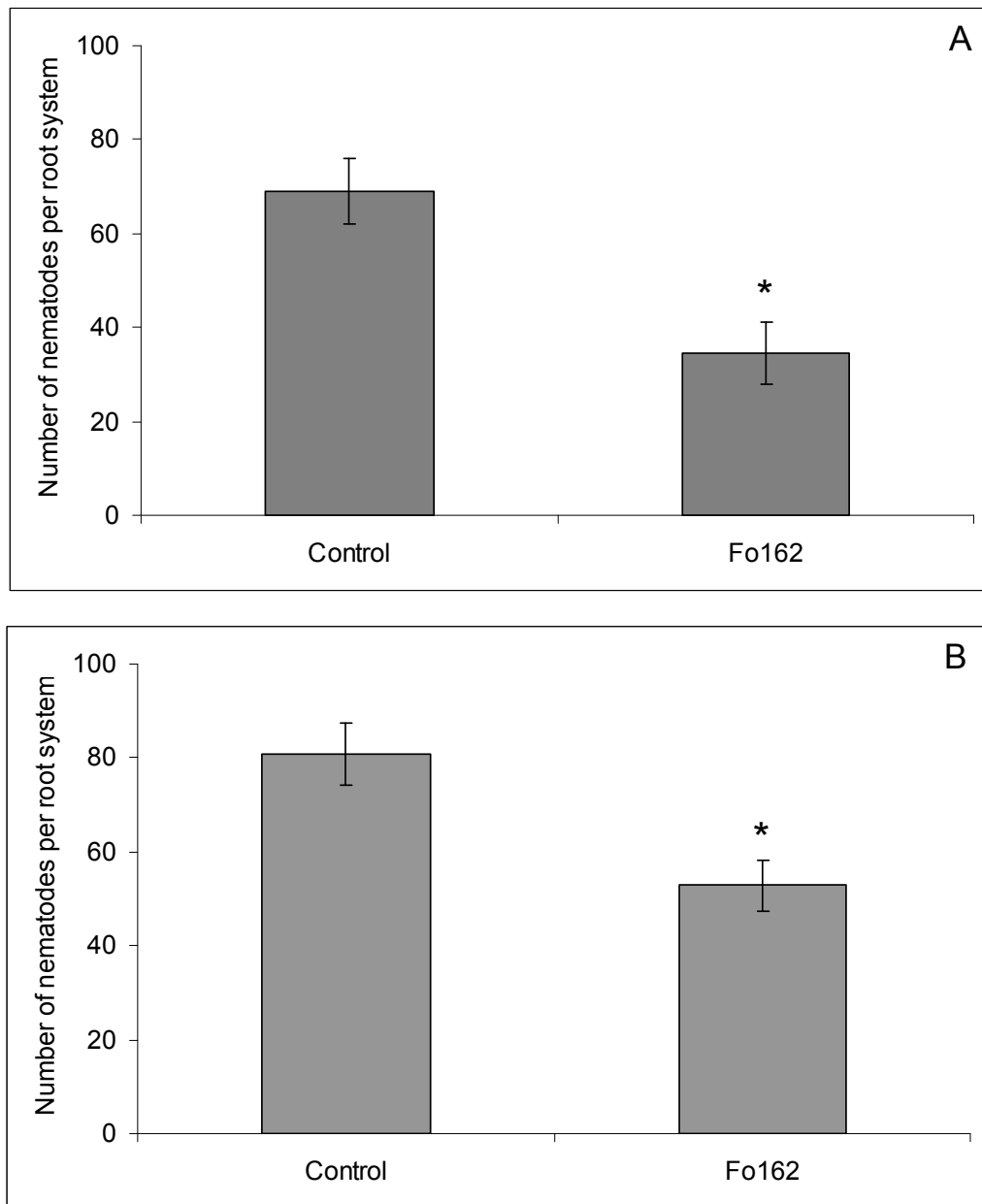
Figure 6.6. Effect of *Fusarium oxysporum* Fo162 on *Meloidogyne incognita* early root penetration in Arabidopsis wild type Columbia. Vertical bars represent standard error of the mean values. Means with (*) indicates significant different after *t*-test for independent samples ($P \leq 0.05$, n=10). A, Experiment 1 and B, Experiment 2.
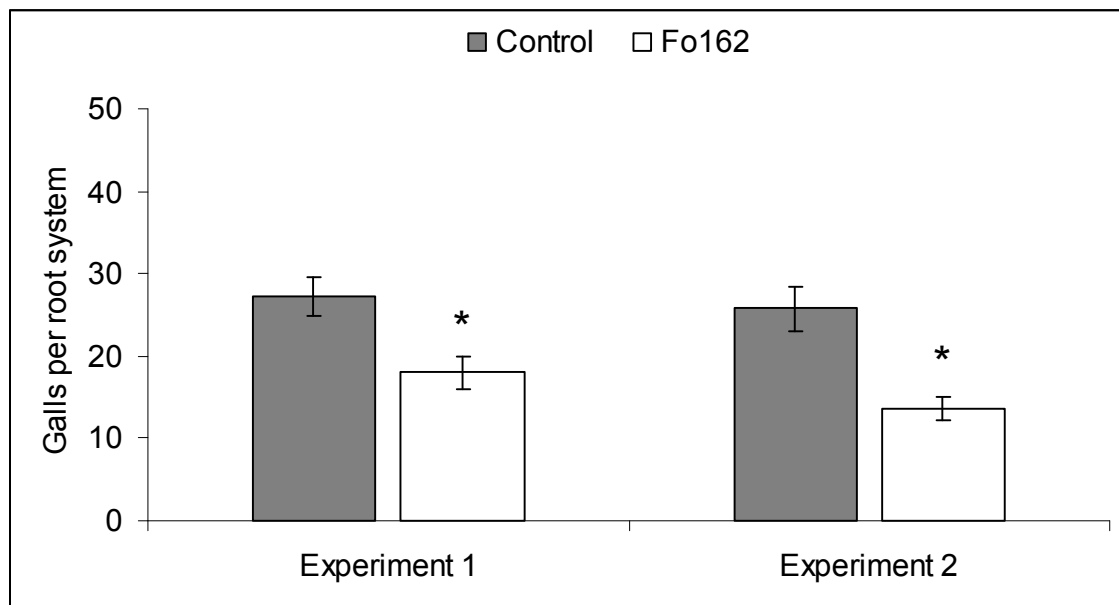
Figure 6.7. Systemically induced resistance of *Fusarium oxysporum* strain Fo162 on *Meloidogyne incognita* galls formation in an Arabidopsis split-root system. Vertical bars represent standard error of the mean values. Means with (*) indicates significant differences after *t*-test for independent samples ($P \leq 0.05$, n=8).

6.3.4. Induction of host plant systemic resistance by different combinations of chemical elicitors on *M. incognita* gall formation

This experiment showed that different combinations of SA and MeJA inhibited *M. incognita* infection on the responder side of the split-root Arabidopsis plants. Twenty days after nematode inoculation, the total number of galls per root system was significantly reduced at the responder side of the Arabidopsis plants treated with 10 μM SA + 10 μM MeJA, 50 μM SA + 10 μM MeJA, 50 μM SA + 250 μM MeJA, 100 μM SA + 0 μM MeJA, at the inducer side of the split-root Arabidopsis plants in Experiment 1 (P=<0.0001) and Experiment 2 (P=<0.0001) (Fig. 6.8, indicated by vertical arrows). In Experiment 1, the reductions in number of galls per root system with respect to control plants (0 μM SA + 0 μM MeJA) were 46, 59, 56 and 54 percent in plants treated with the combinations of 10, 50, 50, 100 μM SA with 10, 10, 250, 0 μM MeJA respectively; while in Experiment 2, the reductions in number of galls per root system for the treatments combinations of 10, 50, 50, 100 μM SA with 10, 10, 250, 0 μM MeJA were 18, 27, 22 and 11 percent respectively.
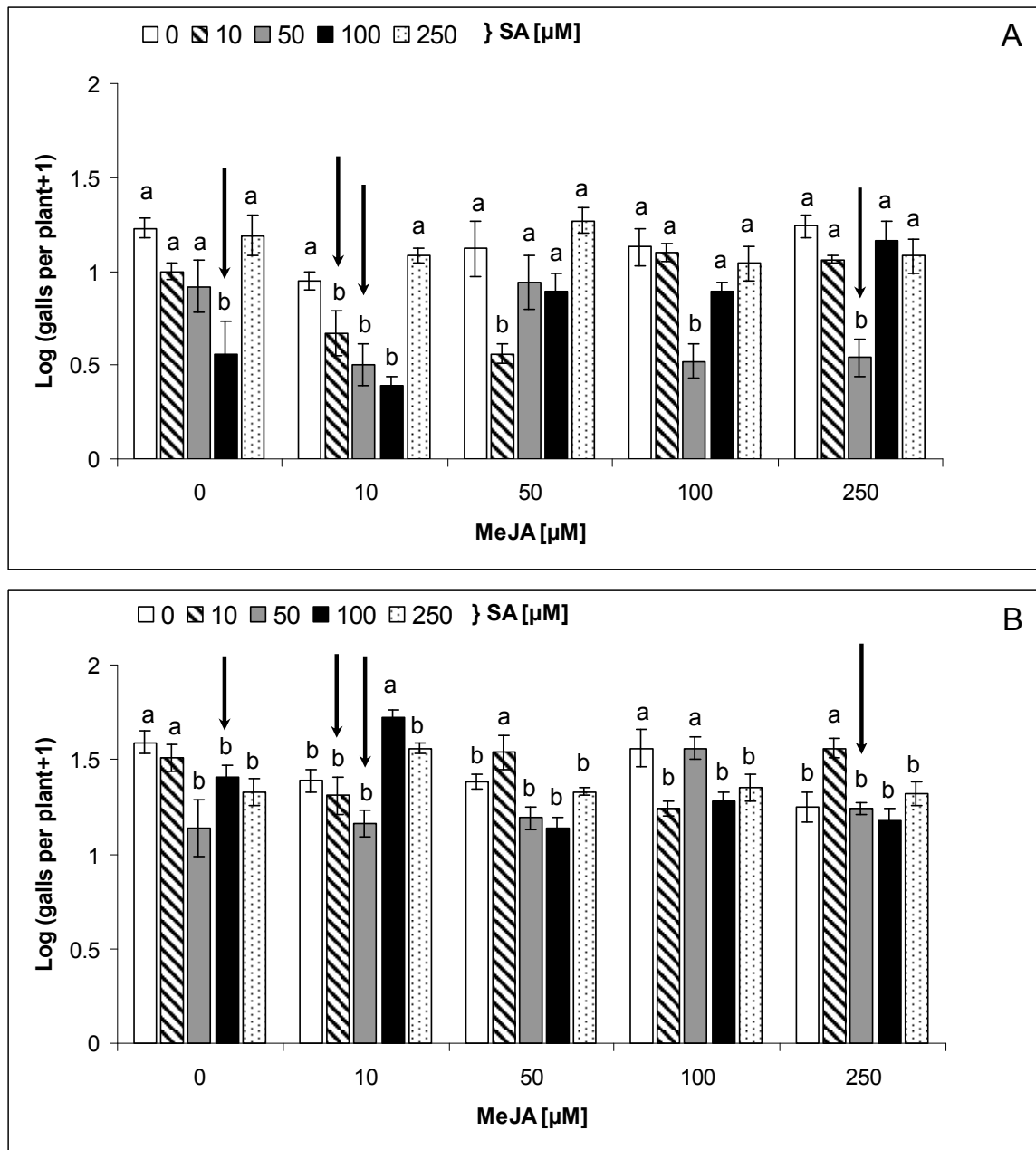
Figure 6.8. Salicylic acid (SA) interactions with methyl jasmonate (MeJA) on *Meloidogyne incognita* galls formation at the responder side of a split-root Arabidopsis plant. The inducer root section was treated with a mixture of 0, 10, 50, 100, 250 μM SA with 0, 10, 50, 100, 250 μM MeJA. Number of galls was counted at the responder side of the split-root system 20 days after nematode inoculation. Data are presented as Log(x+1). Vertical bars represent standard error of the mean. Columns with different letters are significantly different based on the Scott-Knott test ($P \leq 0.05$, n=4). A, Experiment 1 and B, Experiment 2.

6.4. Discussion

The results from the current study demonstrated the ability of *F. oxysporum* Fo162 to successfully colonize Arabidopsis roots, resulting in enhanced plant fresh weight, root length and root diameter. These results on colonization are similar to those reported by Peskan-Berghöfer et al. (2004), who demonstrated the ability of *Piriformospora indica*, an endophytic fungus of the Sebacinaceae family, to colonize Arabidopsis roots and to stimultate shoot fresh weight and root dry weight. Conversely, Epple et al. (1995) reported that wild type Arabidopsis plants grown under lab conditions developed disease symptoms after inoculation with *F. oxysporum* f.sp. *matthiolae*. Also, the wheat pathogens *F. graminearum* and *F. culmorum* were shown to develop disease symptoms on wild type Arabidopsis plants after inoculation of the flowers (Urban et al., 2002). Interestingly, the percentage of Fo162 root colonization was similar to that reported by other authors. Mendoza and Sikora (2009) reported that Fo162 colonized banana roots to a level of 25%, while for the same fungal isolate Menjivar et al. (2011), Selim (2010) and Martinuz and Sikora (2011) reported root colonization rates between 15 to 50% in pepper, melon and tomato.

Arabidopsis plants treated with Fo162 resulted in reduced penetration of *M. incognita* in the root when compared to non-inoculated control plants. These results confirmed the ability of Fo162 to repress *M. incognita* colonization as observed in tests with tomato, melon and pepper (Dababat and Sikora, 2007; Menjivar et al., 2011). The level of nematode biocontrol encountered in the present research is probably the result of a reduction in attractiveness of Arabidopsis to *M. incognita*. This activity may be a form of resistance that may also be induced systemically by Fo162 as reported earlier in tomato (Dababat and Sikora, 2007; Selim, 2010). Dababat (2006), Diedhiou et al. (2003) and Selim (2010) also showed that Fo162 reduced *M. incognita* infestation by preventing juveniles from invading the roots and by interfering with juvenile development within the root tissue.

The results in the present studies also demonstrated the ability of Fo162 to systemically reduce *M. incognita* infection in Arabidopsis in a split-root system. Similar results for the same fungal isolate in tomato was earlier reported by Dababat and Sikora (2007) and

Martinuz and Sikora (2011). In both cases, the presence of Fo162 at the inducer side of a split-root tomato plant resulted in lower juvenile penetration at the responder side.

In split-root experiments, Selim (2010) demonstrated systemically induced resistance against *M. incognita* in tomato after treatment of the inducer half with *F. oxysporum* Fo162, SA or MeJA. The experiment demonstrated that both jasmonic acid (JA)- and SA- dependent signaling pathways were involved in induced resistance towards *M. incognita* in tomato. Therefore, both systemic acquired resistance (SAR) and induced systemic resistance (ISR) may be pathways that are involved in increased plant resistance towards the nematode. By using triple-split-root tomato plants, Martinuz and Sikora (2011) demonstrated that spatially-separated but simultaneous inoculation of two different types of endophytes (*F. oxysporum* Fo162 and *Rhizobium etli* G12) did not lead to additive reductions in *M. incognita* infection. Moreover, spatially-separated inoculation of Fo162 and G12 led to a reduction in fungal root colonization. It was discussed that the suppressive activity of G12 on Fo162 and *M. incognita* is possibly related to negative interactions between the JA- and SA-dependent signaling pathways, preventing therefore an additive effect on the nematode biocontrol.

The cross-talk between the JA- and SA-dependent signaling pathways towards *M. incognita* was tested through a split-root Arabidopsis plant. This experiment demonstrated that co-treatments of various concentrations of SA and MeJA at the inducer side resulted in a reduction in the number of galls per root system at the responder side of the split-root system. This reduction in number of galls was observed when both elicitors were applied at low concentrations.

Mur et al. (2006) in studies investigating gene expression and cell death reported similar results after treating tobacco and Arabidopsis plants with a mixture of various concentrations of SA and JA. They concluded that there was an enhancement in the expression of genes associated with either JA (PDF1.2 and Thi1.2) or SA (PR1) signaling when both signals were applied at concentrations ranging from 10 to 100 μM. Conversely, antagonism was observed at higher concentrations and prolonged treatment times, resulting in cell death.

Overall, the results demonstrated that the Arabidopsis–Fo162 interaction is an ideal model system for research on plant defense responses towards *M. incognita*, in the presence of a mutualistic endophytic fungus. This system opens the possibility to increase our understanding of the molecular basis of microbial induced resistance using a fungus and an animal parasite.

6.5. Conclusions

1. The mutualistic endophytic fungus *F. oxysporum* Fo162 was able to effectively colonize Arabidopsis plants.

2. Arabidopsis plant fresh weight, root length and average root diameter improved after plant treatment with the endophyte.

3. Fo162 caused a significant level of biocontrol activity towards *M. incognita* in Arabidopsis.

4. In a split-root experiment with Arabidopsis and spatially-separated inoculation of both Fo162 and *M. incognita*, a systemic reduction in the number of galls per root system was demonstrated.

5. In a split-root test with Arabidopsis, co-treatments of various concentrations of SA and MeJA at the inducer side resulted in a reduction in the number of galls per root system at the responder side of the system. The interactions between the JA- and SA-dependent signaling pathways therefore are important for the biocontrol of *M. incognita* in Arabidopsis.

6.6. References

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Chapter 7
General conclusions

- The variation in the level of biocontrol, mediated by microbial antagonists, is considered an important disadvantage for commercial applications. This lack of consistent levels of control can be caused by a number of biotic and abiotic factors. For example, the level of colonization by the biocontrol agent is affected by abiotic factors such as moisture, temperature and pH. Colonization can also be affected by competition with other rhizosphere and endosphere organisms for similar habitats in and on the root. Another factor that might affect control is related to the fact that normally only a single microorganism is applied to the soil, seed or seedling and they usually only have one mode of action for control or the target pest or pathogen.

- Therefore, co-inoculation strategies, in which different microorganisms with different mechanisms of action are combined, have been suggested as a means of enhancing biocontrol activity (El-Tarabily et al., 2000; Sikora et al., 2007; Reimann et al., 2008; Sikora et al., 2008; Chaves et al., 2009; Sikora et al., 2010). Combining biocontrol agents could result in additive and possibly even synergistic increases in the level of protection against nematodes or insects as indicated in previous works (Gadelhak et al., 2005; Mendoza and Sikora, 2008; Reimann et al., 2008; Chaves et al., 2009). Whether synergistic control levels can be reached has to date not been shown. Thus, the objective of the present study was to investigate the interrelationship between *Fusarium oxysporum* strain Fo162 (Fo162), *Glomus intraradices* strain 510 (AMF) and *Rhizobium etli* strain G12 (G12) when applied together on infection of *Meloidogyne incognita* and *Aphis gossypii*. This combination includes three organisms from vary different antagonistic groups that are considered to have different colonization behavious and most likely different mode of action.

- In a large array of experiments designed the following effects were examined: 1) the effect of single and dual applications of Fo162 with AMF or G12 on the biocontrol of *M. incognita* in tomato to determined the ability of the antagonists to reduced *M. incognita*

penetration when applied alone or in combination; 2) the ability of Fo162 and G12 to reduce systemically *M. incognita* penetration, when applied to the plant in a spatially-separated manner and 3) the influence of Fo162 or G12 alone on nematode development.

−   As expected, the individual application of each of the biocontrol agents on tomato resulted in a significant reduction in the number of root-knot nematodes penetrating the roots. However, the combined application of both Fo162 and G12 did not cause an additive reduction in nematode penetration. A triple-split-root experiment showed that the simultaneous but spatially separated inoculation of both Fo162 and G12 also did not lead to a significant difference in *M. incognita* penetration when compared to the individual inoculation. More importantly and unexpectedly, a significant reduction in Fo162 root colonization was observed when the plant was colonized simultaneously with G12. This demonstrated for the first time that the colonization of an antagonistic endophytic fungus can be systemically inhibited by an antagonistic bacterium. Additional experiments demonstrated that Fo162 and G12 root colonization reduced the rate of development of the nematode from the second stage juvenile to the adult stage in endophyte treated plants when compared to the untreated controls. In addition, fungal and bacterial endophyte treatments led to a significant reduction in the number of eggs per female.

−   In further investigations, single and dual applications of Fo162 with AMF or G12 were studied on squash to determine the ability of the antagonists to reduced *A. gossypii* populations. Spatially separated dual inoculation of Fo162 and G12 on *A. gossypii* performance also was studied as was the influence of a single application of Fo162 and G12 on *A. gossypii* host preference.

−   The individual application of each of the antagonists on squash resulted in a significant reduction of *A. gossypii* final population. However, concomitant inoculations with Fo162 together with AMF or with G12 did not lead to significantly higher reductions. A split-root experiment showed that the simultaneous but spatially separated inoculation of both endophytes reduced the aphid population in comparison to untreated squash plants.

Nevertheless, the concomitant treatment again did not lead to significant additive nor synergistic additive levels of biocontrol activity with respect to reducing the aphid population when compared to individual inoculation. A pair wise choice experiment with intact squash plants demonstrated that *A. gossypii* preferred to feed on endophyte-free plants than on the endophyte-inoculated plants; when plants inoculated with Fo162 and G12 were paired, the majority of *A. gossypii* was found on G12-inoculated plants.

- The results demonstrated that competition between the antagonistic microorganisms, both in or on the root system, could lead to direct or indirect competition and thereby reduced colonization and/or activity of one or more of the microorganisms being used to enhance biological control efficacy through co-inoculation. The interaction between the fungus Fo162 and the bacteria G12 was then analyzed *in vitro* and *in vivo*. Antibiosis tests showed that there was a significant reduction in radial growth of Fo162 when challenged with G12 and that this reduction in radial growth was positively correlated with the bacterial incubation period. Greenhouse experiments with tomato indicated that simultaneous inoculation of Fo162 and G12 resulted in reduction of Fo162 root colonization, indicating that fungal colonization can be directly inhibited by the presence of the bacteria. In an additional split-root experiment, spatial separation of Fo162 and G12 again resulted in a reduction of Fo162 root colonization, indicating that the fungus can also be systemically inhibited by the bacterium.

- Overall, the results demonstrated that antagonists not only induce resistance to a target pest but can also have side-effects on other organisms present in or on the plant. It also showed the need to test for incompatibility between microbial biocontrol agents when considering a co-inoculation strategy.

- The plant defense responses towards *M. incognita* triggered by the endophyte Fo162 were further studied on the model plant *Arabidopsis thaliana*. The initial experiments analyzed the ability of Fo162 to colonize Arabidopsis roots and its effect on plant growth parameters. In the second set of experiments the ability of Fo162 to reduced *M. incognita* penetration, when applied single or in a spatially-separated way to the same plant, was

studied. Finally, the effect of inducing host plant systemic resistance using different combinations of chemical elicitors on root gall formation of *M. incognita* was examined.

−   The results demonstrated the ability of Fo162 to successfully colonize Arabidopsis roots, resulting in enhanced plant fresh weight, root length and root diameter. Arabidopsis plants treated with Fo162 resulted in reduced penetration of *M. incognita* in the root when compared to non-inoculated control plants. A split-root experiment showed that the simultaneous but spatially separated inoculation of both Fo162 and *M. incognita* lead to a significant reduction in the number of galls per root system when compared to the water control.

−   In a split-root experiment, concomitant treatments of various concentrations of salicylic acid (SA) and methyl jasmonate (MeJA) at the inducer side resulted in a reduction in the number of galls per root system at the responder side of the system. The interactions between the JA- and SA-dependent signaling pathways therefore are important for the biocontrol of *M. incognita* in Arabidopsis. Overall, the results demonstrated that the Arabidopsis–Fo162 interaction is an ideal test system for research on microbial induced plant defense responses towards the root-knot nematode *M. incognita*. This system opens the possibility to increase our understanding of the molecular basis of microbial induced resistance using a fungal antagonists and a highly specialize sedentary plant parasitic nematode.

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