# Biological Control of *Meloidogyne incognita* (Tylenchida: Meloidogynidae) on Tomato Using Arbuscular Mycorrhizal Fungi and Rhizobacteria

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**Biological control of *Meloidogyne incognita* (Tylenchida: Meloidogynidae) using arbuscular mycorrhizal fungi and rhizobacteria.**

The sedentary endoparasitic root-knot nematode, *Meloidogyne incognita*, attacks the majority of the flowering plant species. It poses particular control difficulties due to its wide host range, short generation period and high reproductive rate. The worldwide phase-out of methyl bromide and the extreme costs for bringing new nematicides into the market increases the need for alternative nematode control strategies that are economically feasible and environmentally acceptable. Biological control of nematodes using rhizosphere micro-organisms is considered as a potential management tactic and effective alternative of nematicides. The main focus in this research area has been given to groups of soil micro-organisms co-occurring with plant parasitic nematodes; among those are mycorrhizal fungi and rhizobacteria. However, despite the intensive research, the topic of AMF/nematode interaction is in a greatly confused state. The poor understanding of the mechanisms involved contributes to this situation. Subjects of this study were i) to screen for an effective mycorrhizal isolate that confers bio-protection against *M. incognita* and ii) to characterize the influence of AMF on the different nematode pre- and post-infectional aspects, iii) to elucidate possible involved mechanisms, and iv) to consider physiological markers for the interaction between AMF and *M. incognita*. Moreover, v) the possible stimulation of the mycorrhizal effect against *M. incognita* was tested through the combined inoculation of AMF with bacterial isolates of known effects on *M. incognita*.

Nematode-mycorrhiza interactions appear to be highly dependent on the given association of plant cultivar, nematode species, and AMF species or isolate. Several mycorrhizal isolates were screened; the differences in their efficacy to suppress nematode infection could not be attributed to differences in their ability to colonize the roots or to enhance plant growth. An isolate of *Glomus intraradices* (No. 510) reduced gall numbers induced by *M. incognita* in the roots of the tomato cultivar Kingkong II. The sequence of aspects in the interaction between the two partners were tested. Differences in final number of galls were not attributed to differences in nematode pre-infectional aspects. Root diffusates collected from mycorrhizal and non-mycorrhizal plants did not influence egg hatch of *M. incognita*. Second stage juveniles were less attracted to mycorrhizal plants than to non-mycorrhizal plants in a pair-choice assay. In an invasion assay, second stage juveniles were slower in invading roots of mycorrhizal plants compared to the non-mycorrhizal control. However, the final number of juveniles detected in roots of mycorrhizal plants was

similar as in non-mycorrhizal ones. Nematode suppression is partially attributed to induced resistance mechanisms as observed in split root experiments.

The amount of AMF inoculum that had been added to the substrate initially did not influence the degree of nematode infection suppression; however, the diameter of nematode galls was significantly influenced. Considering proline and the performance index for chlorophyll-*a*-fluorescence ($PI_{abs}$) as physiological markers for the interaction, it was observed that nematode infection caused an increase in proline content of roots, parallel with increasing density of nematode inoculation level. AMF slightly reduced the concentration of proline in roots. Nematode inoculation caused also a decrease of $PI_{abs}$ that declined with raising nematode inoculation levels. At earlier stages of the experiment, AMF inoculation had positive influence on the $PI_{abs}$ state; however, this effectdiminished with the time course of mycorrhizal infection.

Results of combined inoculation of micro-organims suggest that the co-inoculation of tomato with AMF and either bacterium *Cellulomonas turbata* (SR1), or *Acinetobacter baumannii* (SR6) can improve the efficacy of *M. incognita* control conferred by the single inoculation of the AMF. The mycorrhizal symbiosis had no influence on the bacterial population density and itself was not influenced by the bacteria.

Overall, an isolate that confers a bio-protective activity against *M. incognita* had been selected and the characterization of the influence of AMF on the different nematode pre- and post-infectional aspects revealed that the attraction of juveniles was sensitive to the plant mycorrhizal state. It appeared that reduction in nematode infection can be attributed to post-infectional aspects and may be due in part to induced resistance. It was shown that combined inoculants of AMF and certain rhizobacteria provided a more stable control and that the binary association of bacteria and mycorrhizal fungi could be beneficial to plant health and growth.

Keywords: *Meloidogyne incognita*, Biological control, arbuscular mycorrhizal fungi, rhizobacteria.

**Biomanagement von *Meloidogyne incognita* (Tylenchida: Meloidogynidae) an Tomate mit Hilfe von arbuskulären Mykorrhizapilzen (AMF) und Rhizobakterien**

Der sedentäre Wurzelgallen-Nematode *Meloidogyne incognita* befällt sehr viele Arten der Blütenpflanzen. Bedingt durch diesen weiten Wirtspflanzenkreis, eine kurze Generationszeit und eine hohe Reproduktionsrate treten besondere Schwierigkeiten bei der Bekämpfung auf. Der weltweite Rückzug von Methylbromid einerseits und die extremen Kosten für die Entwicklung und Vermarktung neuer Nematizide andererseits erhöhen den Bedarf an alternativen Strategien zur Bekämpfung von Nematoden, die sowohl ökonomischen realisierbar als auch ökologisch vertretbar sind. Die biologische Bekämpfung von Nematoden durch Rhizosphären-Mikroorganismen wird als potentieller Lösungsweg und Alternative zu chemischen Nematiziden betrachtet. Der Schwerpunkt in diesem Forschungsbereich lag auf verschiedenen Gruppen von Boden-Mikroorganismen, die gemeinsam mit pflanzenparasitären Nematoden vorkommen; zu diesen Gruppen zählen auch Mykorrhizapilze und Rhizobakterien. Trotz intensiver Forschung liegen zur Thematik der AMF-Nematoden-Interaktionen bisher widersprüchliche Thesen vor. Insbesondere fehlen Erkenntnisse über Mechanismen der Wechselwirkungen. Themen dieser Arbeit waren i) die Auswahl eines effektiven Mykorrhiza-Isolates mit biologischer Schutzwirkung gegen *M. incognita*, ii) die Charakerisierung des Einflusses von AMF auf den Nematoden in verschiedenen prä- und postinfektionellen Abschnitten, iii) die Aufklärung möglicher beteiligter Mechanismen und iv) die Überprüfung physiologischer Marker für diese Beziehung. Darüberhinaus sollte v) eine mögliche Stimulation des Mykorrhizaeffektes durch Dualapplikation von AMF und Rhizobakterien mit bekannter Wirkung gegen *M. incognita* getestet werden.

Nematoden-Mykorrhiza-Interaktionen sind hochgradig abhängig von der vorliegenden Kombination von Pflanzensorte, Nematodenart und AMF-Art oder Isolat. Verschiedene Mykorrhiza-Isolate wurden getestet; ihre unterschiedliche Wirksamkeit zur Unterdrückung der Nematodeninfektion konnte nicht zurückgeführt werden auf ihre unterschiedliche Fähigkeit zur Wurzelbesiedlung und zur Steigerung des Pflanzenwachstums. Das Isolat 510 von *Glomus intraradices* reduzierte die Anzahl der von *M. incognita* induzierten Gallen in der Tomatensorte Kingkong II. Verschiedene Aspekte in der Folge der Interaktionen beider Partner wurden untersucht. Der Unterschied in der Endzahl an Gallen konnten nicht korreliert werden mit unterschiedlichem präinfektionellem Verhalten des Nematoden. Wurzeldiffusate von mykorrhizierten und nicht-mykorrhizierten Pflanzen beeinflussten den Eischlupf von *M. incognita* nicht.

In einem Wirtswahlversuch wurden Larven von mykorrhizierten Pflanzen weniger angezogen als von nicht-mykorrhizierten. In einem Besiedlungsversuch drangen Larven langsamer in die Wurzeln mykorrizierter Pflanzen ein als in die nicht-mykorrhizierter Kontrollen. Die Endzahl der Larven in mykorrhizierten Pflanzen unterschied sich jedoch nicht von der in nicht-mykorrhizierten Pflanzen. Die Abwehr von Nematoden ist teilweise induzierten Resistenzmechanismen zuzuschreiben, wie sie in einem Split-Root-Experiment beobachtet wurden.

Die Menge des applizierten AMF-Inokulums beeinflusste nicht den Wirkungsgrad der Unterdrückung von Nematodeninfektionen, jedoch war der Durchmesser der Nematodengallen signifikant reduziert. Unter Zuhilfenahme der physiologischen Marker Prolingehalt und Performance Index der Chlorophyll-$\alpha$-Fluoreszenz ($PI_{abs}$) wurde beobachtet, dass mit steigender Inokulumdichte die Nematodeninfektionen einen Anstieg des Prolingehalts der Wurzeln verursachten. AMF reduzierten geringfügig den Wurzel-Prolingehalt. Unter Nematodenbefall fiel der Performance Index $PI_{abs}$ mit zunehmender Inokulumdichte ab. Zu Beginn dieses Experiments wirkte sich die Mykorrhiza positiv auf den $PI_{abs}$ aus; dieser Effekt verlor sich im Verlauf des Versuches.

Die Ergebnisse von kombinierter Inokulation (AMF mit Bakterium *Cellulomonas turbata* (SR1) oder *Acinetobacter baumannii* (SR6)) deuteten darauf hin, dass die Wirksamkeit von AMF allein gegen *M. incognita* auf diese Weise gesteigert werden kann. Die Mykorrhiza hatte keinen Einfluss auf die Populationsdichte der Bakterien und war selbst durch die Bakterien nicht beeinträchtigt.

Zusammengefasst: Es wurde ein Isolat mit bioprotektiver Aktivität gegen *M. incognita* ausgewählt und sein Einfluss auf prä- und postinfektionelle Beziehungen zum Nematoden untersucht. Es ergab sich, dass die Attraktion infektiöser Larven vom Mykorrhiza-Status der Wirtspflanzen abhängt. Ferner kann die Reduktion der Nematodeninfektionen verknüpft werden mit postinfektionellen Wechselwirkungen und partiell auf induzierter Resistenz beruhen. Es zeigte sich, dass die Kombination von AMF und bestimmten Rhizobakterien eine solidere Nematodenbekämpfung ermöglicht und sich auch positiv auf Pflanzengesundheit und Pflanzenwachstum auswirkt.

Schlagworte: *Meloidogyne incognita*, Biologische Bekämpfung, arbuskulären Mykorrhizapilzen, Rhizobakterien.

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## Abbreviations

| | |
|---|---|
| a. dest. | Distilled water |
| AMF | Arbuscular Mycorrhizal Fungi |
| cfu | Colony Forming Units |
| dai | Days after nematode inoculation. |
| g | gram |
| $g_{av}$ | Acceleration of free fall |
| Ge | *Glomus etunicatum* |
| Gi | *Glomus intraradices* |
| IR | Inner root periphery |
| J2 | Second stage juveniles (of *M. incognita*) |
| l | liter |
| MI | Mycorrhizal infection. |
| MF | Frequency of mycorrhizal colonization |
| Mi | *Meloidogyne incognita* |
| ml | millilitre |
| µl | microliter |
| µg | microgram |
| No. | Number |
| OR | Outer root periphery |
| PCR | Polymerase Chain Reaction |
| ppm | parts per million |
| R L | Root length |
| RKN | Root-knot nematode |
| sec. | Second |
| Sh DWT | Shoot dry weight |
| Sh FWT | Shoot fresh weight |
| SR1 | *Cellulomonas turbata* |
| SR1** | SR1 resistant to 100 ppm rifampicin and streptomycin |
| SR6 | *Acinetobacter baumannii* |
| SR6** | SR6 resistant to 100 ppm rifampicin and streptomycin |
| Wks | Weeks |

# 1 General introduction

Soil is a dynamic microbiologically complex environment. A single cubic meter would contain hundreds of species of micro-organisms whose identities, quantities and activities are largely unknown (Copley, 2000). In the course of their existence, plant pathogenic and non-pathogenic micro-organisms come into association with each other; the outcome of this association may involve interactions among the different groups.

Rhizosphere is the site where most interactions between plants and their subterraneous environment occur. The outcomes – from plant's perspective – are many and range from harmful to beneficial and would likely change when the interactors or when the surrounding environmental factors alter. Symbioses, parasitism, and antagonism, are part of an array of interactions that would take place in the rhizosphere.

Since the 1800s agricultural soils have been degraded due to cultivation, monoculture and excessive chemical inputs. The chemical components to support plant growth have been sustained over decades through fertilizer application. However, the maintenance of the balanced state of agricultural soils exceeds the replacement of nutrients consumed by the crops to the maintenance of the chemical, physical and biological components of soil (Bethlenfalvay and Linderman, 1992). The underlying processes and functions in soils- such as mineralization, nitrification, nitrogen fixation, and plant biodiversity- are governed by soil micro-organisms; loss of some species may result in a loss of some soil functions (van der Heijden *et al.*, 1998; Griffiths *et al.*, 2000; Emmerling *et al.*, 2002). Managing the positive attributes of micro-organisms in agricultural soils does not only affect soil fertility but is likely to carry the key to improved suppression of soil borne pathogens, especially in light of the increased costs of chemical pesticides and the increased concern of environmental pollution.

Biological control of soil-borne pathogens by introduced micro-organisms has been studied for over 60 years (Barker, 1987), and considered to be a potential non-chemical mean of plant disease control (Spiegel and Chet, 1998). Initially, plant pathologists adopted the entomologist's classical definition of biological control where the emphasis is on the use of predaceous or parasitic organisms to maintain another organism's density at a lower average than that would occur in their absence (Wilson, 1997). In the

area of biological control of diseases caused by soil-borne pathogens the goal is to reduce the inoculum potential of the pathogens in soil. The fundamental difference between the objects to be controlled by entomologists and plant pathologists is that entomologists are targeting an organism (the insect), while plant pathologists are targeting a process (the disease) and an organism (the pathogen), strategies controlling the disease process (therapy) can differ from those used to control the pathogen (Wilson, 1997). Focus in this research area has been given to the major groups interacting with land flora; amongst those, mycorrhizal fungi and rhizobacteria.

Mycorrhizal associations have existed since at least 350 million years (Simon *et al.*, 1993; Taylor *et al.*, 1995). They have been found with plants in polar, temperate and tropical areas (Mosse *et al.*, 1981). Mycorrhizas vary widely in structure and are classified into different types according to their morphological relationships with the host plants. Two broad types are predominant, endomycorrhiza and ectomycorrhiza, with the endomycorrhiza further divided into vesicular arbuscular, ericoid, and orchidaceous mycorrhizas (Mitchell, 1993).

Arbuscular mycorrhizal fungi (AMF), by far the most widespread mycorrhizal fungi, constitute a low diversity taxon, with approximately 150-160 species being known world wide (Brussaard *et al.*, 2001). AM fungi belong to the Glomeromycota (Zygomycotina) (Schuessler *et al.*, 2001) under which seven genera are classified; Glomus, Entrophospora, Acaulospora, Gigaspora, Scutellospora, Archaeospora and Paraglomus. Arbuscules are the common character in the symbioses formed by this group of fungi. Among these genera; Glomus is the most widespread, it comprises about 90 species.

AMF colonize endogenously the epidermis and the cortical parenchyma of the root, forming intimate connections with the cells of more than 80% of vascular plant taxa, including Bryophyta, Pteridophyta, Gymnospermae and Angiospermae which reflect a wide host range for each individual fungal species and consequently a low degree of taxonomic specificity. Reports about AMF effects on their host plants indicate that the outcomes are highly specific regarding species or isolate of AMF used. Despite the lack of host specificity and the wide host range of individual AMF, there is a level of functional compatibility shown by symbioses between different AM isolates and

different plant species or cultivars (Harrier and Watson, 2004), this has been addressed (Newsham *et al.*, 1995; Van der Heijden and Kuyper, 2001) in three respects: Different effects of different fungi on the same plant; different effects of the same fungus on different plants; different effect of the same fungus on the same plant under different environmental conditions. However, the knowledge concerning how and why fungal species or isolates differ in their symbiotic efficiency remains very limited (Varma, 1998).

The ecological significance of AMF is being intensively studied because of their ubiquitous nature, their influence on plant diversity and productivity, and also due to their ability to protect their hosts from various stresses. AMF are recognized as an ecologically important group of organisms, which contributed to the maintenance of plant biodiversity and to ecosystem functioning and are considered important in maintaining a basic level of plant biodiversity (van der Heijden *et al.*, 1998), the authors emphasized that even at low AMF diversity, an alteration in the composition and number of AMF taxa can lead to fluctuations in the composition of plant communities.

The most thoroughly studied benefit of the mycorrhiza is the growth stimulation of the host plant. Enhanced plant growth following mycorrhizal colonization was demonstrated for a wide variety of plant species (Mosse, 1973; Smith and Gianinazzi-Pearson, 1988; Koide, 1991). The effect of AMF on plant growth can be either direct or indirect. AMF can play a key role in plant growth by increasing acquisition of low mobility nutrients such as phosphate, zinc and copper (Evans and Miller, 1988; Gnekow and Marschner, 1989; Smith and Read, 1997; Karagiannidis and Hadjisavva-Zinoviadi, 1998; Cantrell and Linderman, 2001). A variety of mechanisms and symbiotic effects have been suggested to be involved in improved nutrient uptake by mycorrhizal plants. Firstly, increased total root length – and consequently increased absorbing surface - would contribute to increased total uptake. Secondly, extraradical hyphae of mycorrhizal fungi reduce the distance that nutrients must diffuse to plant roots and increase the volume of accessible soil. Thirdly, mycorrhizal hyphae may physically and/or chemically modify the availability of nutrients for uptake. In addition to the direct influence on plant growth due to improved nutrient status, the presence of AMF also impart other benefits to plants as improved soil aggregation and thus improved soil physical properties and stability (Bethlenfalvay and Linderman, 1992). They increase

plant tolerance to abiotic stress such as chilling (Charest *et al.*, 1993), drought (Kothari *et al.*, 1990) and salinity (Cantrell and Linderman, 2001).

Apart from their influence on plant biodiversity and growth stimulation, AMF contribute to the protection of the host plant against soil borne pathogens. Research considering the bioprotective effects of AMF started in the seventies, their prophylactic effects have been demonstrated for many agronomically important nematodes (Roncadori and Hussey, 1977; Kellam and Schenk, 1980; Pinochet *et al.*, 1996; Jaizme-Vega *et al.*, 1997; Habte *et al.*, 1999; Calvet *et al.*, 2001; Elsen *et al.*, 2001; Talavera *et al.*, 2001; Diedhiou *et al.*, 2003; Elsen *et al.*, 2003), fungal pathogens (Davies and Menge, 1980; Dugassa *et al.*, 1996; Trotta *et al.*, 1996; Rabie, 1998), bacterial pathogens (Rosendahl, 1985; Pardeep and Sood, 2002), plant viruses (Deokar and Sawant, 2001) and insects (Vicari *et al.*, 2002) . Nevertheless, the degree of response of mycorrhizal plants to a certain biotic or abiotic factor is not always the same, since it depends on the specific fungal-host interaction. Conflicting results have been obtained and inconsistencies are reported in some reviews (Dehne, 1982; Todd *et al.*, 2001; Ryan *et al.*, 2003). The high biodiversity and complexity of relationships between the many microbial taxa in soil leads to undefined experimental conditions and contribute to the low reproducibility of observations and some of the contradictory results.

Bioprotection of mycorrhizal plants is an outcome of complex interactions between plants, pathogens and AM fungi (Harrier and Watson, 2004). Therefore, the variability of results concerning the bioprotective ability of AMF could be ascribed to one or a combination of those elements contributing to the final balance of the plant/AMF/pathogen interaction, (1) the AMF isolate, (2) the pathogen, concerning both virulence and inoculum potential, (3) the host plant, (4) the substrate, and (5) the prevailing environmental conditions (Azcón-Aguilar *et al.*, 2002). Apart from these elements, the different methods used for recording disease patterns -which do not always reflect a correlation between the different parameters (Kjøller and Rosendahl, 1996)- contribute to the controversy in literature.

Though much attention that has been devoted to the role of AMF in controlling plant diseases, the mechanisms involved are not yet well characterized. However, bioprotection is more likely to include indirect mechanisms since AMF have not been shown to interact directly with pathogens through antagonism, antibiosis and/or mycoparasitism (Harrier and Watson, 2004). Azcón-Aguilar *et al.* (2002) argued that

the effective bioprotection against root pathogens conferred by AMF is probably a consequence of several, and likely interacting mechanisms; and their relative contribution in the overall protection process is directly related to AMF/plant genotype combination and with the environmental conditions. Literature (Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004) proposed several mechanisms to explain the bioprotection conferred by AMF: (1) Improved nutrient status of the host, (2) damage compensation, (3) anatomical change of the root system, (4) root architecture, (5) competition for colonisation and infection sites, (6) competition for photosynthates, (7) rhizosphere deposition, (8) change on the soil microbial populations and (9) and activation of plant defence responses.

Despite the many studies of the interaction between AMF and soil borne diseases it is still not possible to quantify the impact of single mechanisms on plant health via clear-cut conclusions. To make the situation more complex, the impact on plant growth is not correlated with bioprotective abilities, or with colonization levels. This in turn underlines the need for screening according to defined selection traits (Azcón-Aguilar *et al.*, 2002) to find the appropriate experimental AMF, and emphasizes the need of multidisciplinary approaches to identify their functional diversity and differences in their symbiotic abilities (Varma, 1998).

Plant parasitic nematodes, are among the most widespread and important pathogens causing crop loss. The annual loss in agriculture has been estimated as US $ 100 billion worldwide (Oka *et al.*, 2000). Based on their parasitic strategies, root parasitic nematodes can be classified into five major types: migratory ectoparasites; sedentary ectoparasites; migratory ecto-endoparasites; migratory endoparasites and sedentary endoparasites, with the latest considered as the most economically important group of plant parasitic nematodes (Sijmons *et al.*, 1994).

The sedentary endoparasitc root-knot nematodes, *Meloidogyne* spp., attack the majority of the estimated 250,000 flowering plant species (Sasser and Freckman, 1987). They belong to the order Tylenchida, suborder Tylenchina, superfamily Heteroderoidea; family Meloidogynidae (Dropkin, 1989). Jepson (1987) described 51 species in the genus *Meloidogyne*. Four species; *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, account for 95% of all root-knot nematode infestations in agricultural land

with *M. incognita* being the most economically important species (Sasser and Carter, 1985; Hussey and Janssen, 2003), as it is able to reproduce in more than 2000 plant species (Jung and Wyss, 1999). Studies on host range differences, cytology and mode of reproduction resulted in the classification of several host and cytogenetic races for each of the major four species of *Meloidogyne* (Sasser and Carter, 1985; Trudgill and Block, 2001).

Infection is performed by second stage juveniles, which hatch spontaneously from eggs, or due to stimuli from root diffusates in some instances (Viglierchio and Lownsbery, 1960; Gaur *et al.*, 2000). The juveniles migrate toward roots in response to stimuli emanating from roots, when they get into contact with a root they primarily enter directly behind the root cap; however, penetration can occur at other regions (Hussey, 1985). They migrate then intercellularly between cortical cells, first towards the root tip where they turn to reach the vascular cylinder (Wyss *et al.*, 1992), this process appears to include both mechanichal force and enzymatic secretions from the nematode (Williamson and Hussey, 1996). Shortly after penetration the second stage juveniles reside in the cortical tissue with their heads in the periphery of the vascular tissue. They induce then sophisticated feeding sites, called “Giant cells”. Giant cells arise by expansion of individual parenchyma cells in the vascular cylinder, they undergo rounds of synchronous nuclear division uncoupled from cytokinesis (Bird and Bird, 2001), and they have dense cytoplasm and thickened walls remodelled to form elaborate ingrowths. The parenchyma and pericycle cells embedding the giant cells undergo hyperplasia while the surrounding cortical cells become hypertrophied giving rise to the characteristic root gall (Hussey, 1985). Feeding site inductive signals are believed to be emanated from the nematode, specifically from the pharyngeal glands, they interact with host genes and function as transcription factors (Bird and Bird, 2001). Gene expression patterns within the giant cells are altered during feeding site induction and in mature giant cells (Opperman *et al.*, 1994; Van der Eycken *et al.*, 1996; Bird and Bird, 2001; Wang *et al.*, 2003). Several genes have been identified. The integrity and maintenance of the giant cells is dependent on the continuous stimulus by the nematode (Bird, 1962), however; whether this stimulus is a physiological effect caused by the metabolic sink of feeding or more specific factors is unknown (Bird and Bird, 2001). After the induction of the feeding site the second stage juvenile then molts a second and third time and develops into an adult male or female, the male emerges from the root

while the female remains attached to its feeding site, and it produces eggs released on the root surface in a gelatinous matrix (Dropkin, 1989).

As parasites, nematodes affect plant growth and consequently productivity by disrupting physiological processes of the host (Melakeberhan and Webster, 1993). The damage caused by root knot nematodes results from water stress due to nematode parasitism and the disruption of root vascular tissue at the feeding sites which results in restricted water flow (Meon *et al.*, 1978). Nematode infection causes deformation of the root system, which subsequently prevents roots from extending into moist soil (Hussey, 1985). The physiological effects of the decreased water availability include decreased nutrient uptake and translocation of solutes (Melakeberhan and Webster, 1993). Beside their effect on host-water relationship, several studies report negative effects on the photosynthetic rate (Loveys and Bird, 1973; Wallace, 1974; Melakeberhan and Ferris, 1989; Melakeberhan *et al.*, 1990). However, this effect varies with inoculum levels of the nematodes and duration of infection (Melakeberhan *et al.*, 1986). Apart from being serious pests by themselves, root-knot nematodes may predispose plant roots to subsequent disease attack. Enhanced susceptibility for fungal or bacterial pathogens was reported (Powell, 1971; van Gundy *et al.*, 1977; Deberdt *et al.*, 1999).

Root-knot nematodes pose particular control difficulties due to their wide host ranges, short generation periods and high reproductive rates (Trudgill and Block, 2001). Control measures of plant parasitic nematodes can be classified into chemical, cultural and biological (Trudgill *et al.*, 1992). Conventional control options of nematodes are getting more limited, especially with the increased environmental concern.

Since chemical nematicides were first developed, they played a dominant role in nematode control in major crops (Minton and Baujard, 1990). Generally, nematicides are classified into fumigants and non-fumigants. Soil fumigation is considered as the most common mean used to achieve economical control in agricultural land, and fumigants are considered more effective in controlling root-knot nematodes and in increasing crop yield than the non-fumigant nematicides; due to their broad spectrum activity (Lamberti, 1979; Netscher and Sikora, 1990).

The most effective and widely used fumigant is methyl bromide (Oka *et al.*, 2000). The reason behind the excellent nematicidal activity of methyl bromide is its ability to diffuse in a vapour state within plant roots and kill nematodes and eggs surviving within galls or cysts (Giannakou and Karpouzas, 2003). However, being one of the substances implicated in the depletion of the ozone layer (Ristaino and Thomas, 1998), included methyl bromide in international treaties restricting its availability since January 2001 and calling for the complete ban of this substance in the developed countries by January 2005 (Giannakou and Karpouzas, 2003; Schneider *et al.*, 2003). Chemical control by non fumigant nematicides, as organophosphates and carbamates, is expected to increase after the withdrawal of methyl bromide – with the consequence of new environmental concerns (Oka *et al.*, 2000). Moreover, many of the currently available nematicides offer no long-term suppression, often costly; having differential effects on species of the nematodes and their activity is affected by many environmental factors (Schmitt, 1986; Starr *et al.*, 2002). Developing any new marketable nematicide is a long and expensive process (Schmitt, 1986); reports state that no new widespread use nematicide has been developed in the past 20 years (Starr *et al.*, 2002). The worldwide phase-out of methyl bromide and the extreme cost for bringing new nematicides into the market increases the need for alternative nematode control strategies that are economically feasible and environmentally acceptable – even if these strategies cannot compare to the 100% efficacy of methyl bromide.

Cultural measures to control plant parasitic nematodes include means like crop rotation and soil management (Trudgill *et al.*, 1992) such as soil solarization (Nico *et al.*, 2003) and flooding which had been considered as potential effective cultural practices; however, they are adaptable only in certain regions. Crop rotation may provide a short-term suppression of nematode population densities (Starr *et al.*, 2002). However, due to the polyphagous nature of the pest, as well as the relatively low economic value of some recommended rotational crops, control of root-knot nematodes by crop rotation is very limited (Netscher and Sikora, 1990; Waceke *et al.*, 2001). Host resistance to nematodes can be an effective management tool that complements crop rotation and improves the ease with which effective rotation systems can be developed (Starr *et al.*, 2002). Resistance to several root-knot nematode species is present in some crops such as tomato and soybean; the most widely used and investigated is the *Mi* gene in tomato, which was introgressed from the wild tomato species *Lycopersicon peruvianum*

(Williamson and Hussey, 1996; Trudgill and Block, 2001; Hussey and Janssen, 2003). However, resistance mediated by *Mi* gene is lost at elevated temperature (Dropkin, 1969; Augustin *et al.*, 2002); and races breaking resistance have been found in *M. incognita*, *M. javanica* and *M. arenaria* (Starr *et al.*, 2002). Moreover, virulence against *Mi* gene can develop in some cases after as few as five plantings (Noling, 2000).

Biological control of nematodes using rhizosphere micro-organisms was considered in several reviews to be a potential management tactic and effective alternative of nematicides (Sikora, 1992; Kerry, 1993; 2000; Barker, 2003). The contribution to the biocontrol of plant parasitic nematodes was reported for a great diversity of micro-organisms including: plant growth promoting rhizobacteria (Becker *et al.*, 1988; Verdejo and Jaffee, 1988; Spiegel *et al.*, 1991; Racke and Sikora, 1992; Siddiqui and Ehteshamul-Hauque, 2001; Siddiqui and Shaukat, 2003), bacterial parasites (Singh and Dhawan, 1994), obligate fungal parasites and facultative fungal parasites (Leij *et al.*, 1993, Kok and Papert, 2002), competitors including both fungal endophytes (Hallmann and Sikora, 1994; Diedhiou *et al.*, 2003) as well as mycorrhizal fungi (Roncadori and Hussey, 1977; Kellam and Schenk, 1980**;** Pinochet *et al.*, 1996; Jaizme-Vega *et al.*, 1997; Habte *et al.*, 1999; Calvet *et al.*, 2001; Elsen *et al.*, 2001; Talavera *et al.*, 2001; Todd *et al.*, 2001; Waceke *et al.*, 2001; Diedhiou *et al.*, 2003; Elsen *et al.*, 2003). Although biological control of nematodes using rhizosphere micro-organisms could be a promising approach to suppress those pests, the problems associated with biocontrol in the rhizosphere under practical conditions are far from being totally overcome mainly because of too many species and races occurring naturally. With the current knowledge it is difficult to promote or establish a micro-flora in soils that effectively suppresses nematode population densities, especially in the relatively short period of time of a single growing season (Starr *et al.*, 2002). The major focus in the research regarding biological control of nematodes has been given to the major groups of soil micro-organisms co-occurring with plant parasitic nematodes; among those are mycorrhizal fungi. AMF and root-knot nematodes (RKN) share a striking feature, which is their ability to form associations with the roots of the majority of plant species whereas other biotrophs generally show a restricted host range (Trudgill and Block, 2001). However, despite the intensive research, the topic of AMF/nematode interaction is in a greatly confused state, with a variety of isolated observations from which no useful

generalization could be made (Varma, 1998). The poor understanding of the mechanisms involved contributes to this situation.

The impact of AMF on different host plants and nematode species is presented in table 1.1.

**1.2 Aims of the study**

The main aims of this thesis were i) to screen for isolates of arbuscular mycorrhizal fungi (AMF), effective in suppressing the root-knot nematode (RKN) *Meloidogyne incognita*, ii) to differentiate the aspects in the interaction process, iii) to examine possible mechanisms of action involved in RK control, iv) to use chlorophyll-*a*-fluorescence and proline accumulation as physiological indicators for the interaction between AMF and RKN, and v) to examine the possible existence of synergistic interactions between AMF and plant health promoting rhizobacteria (PHPR) with the aim to increase the stability and efficacy of the biocontrol conferred by AMF.

Table 1.1: Effects of interactions between AMF and plant parasitic nematodes (Nem) on host plant, mycorrhizal symbioses, and nematode species associated as reported in literature (+: positively affected; -: negatively affected; 0: not affected; and /: not reported).

| **Host** | **Nematode** | **Mycorrhizal fungus** | **Separate effect on host by** | | **Dual inoculation effect on** | | | **Author (s)** |
|---|---|---|---|---|---|---|---|---|
| | | | **Nem** | **AMF** | **Host** | **AMF** | **Nem** | |
| Cotton (2 cultivars) | *Meloidogyne incognita* | *Gigaspora margarita* | - | + | + | 0 | 0,+ | Roncadori & Hussey, 1977 |
| Soybean | *Meloidogyne incognita* | *Glomus macrocarpus* | / | / | + | 0 | - | Kellam & Schenk, 1980 |
| Onion | *Meloidogyne hapla* | *Glomus fascilculatum* | - | + | 0 | 0 | 0 | MacGuigwin *et al.*, 1985 |
| Tomato | *Meloidogyne hapla* | Mixture of 4 fungi | - | + | + | 0 | - | Cooper & Grandison, 1986 |
| Cotton | *Meloidogyne incognita* | *Gigaspora margarita* | - | + | + | - | / | Smith *et al.*, 1986 |
| | | *Glomus intraradices* | - | + | + | - | -,0 | |
| Soybean | *Meloidogyne incognita* | *Gigaspora margarita* | / | + | / | 0 | - | Carling *et al.*, 1989 |
| | | *Glomus etunicatum* | / | + | / | 0 | - | |
| Cowpea | *Rotylenchulus reniformis* | *Glomus fascilculatum* | - | + | + | 0 | + | Lingaraju & Goswami, 1993 |
| Banana | *Meloidogyne incognita* | *Glomus mosseae* | - | + | + | 0 | - | Jaizme-Vega *et al.*, 1997 |
| Tomato | *Meloidogyne incognita* | *Glomus mosseae* | - | + | + | / | - | Parvatha Reddy *et al.*, 1998 |
| Ruber tree | *Meliodogyne exigua* | *Gigaspora* spp. | - | + | / | - | / | Schwob *et al.*, 1999 |

Table 1.1 continued: Effects of interactions between AMF and plant parasitic nematodes (Nem) on host plant, mycorrhizal symbioses, and nematode species associated as reported in literature (+: positively affected; -: negatively affected; 0: not affected; and /: not reported).

| **Host** | **Nematode** | **Mycorrhizal fungus** | **Separate effect on host by** | | **Dual inoculation effect on** | | | **Author (s)** |
|---|---|---|---|---|---|---|---|---|
| | | | **Nem** | **AMF** | **Host** | **AMF** | **Nem** | |
| Tomato | *Meloidogyne incognita* | *Glomus mosseae* | - | 0 | -,+ | / | 0,- | Talavera *et al.*, 2001 |
| Carrot | *Partylenchus penetrans* | *Glomus mosseae* | - | 0 | + | 0 | - | Talavera *et al.*, 2001 |
| Pyrethrum | *Meloidogyne hapla* | *Glomus* spp. | | - | | | | Waceke *et al.*, 2001 |
| | | LM1 | + | | + | 0 | - | |
| | | ML34 | 0 | | 0 | - | 0 | |
| | | ML35 | 0 | | + | - | 0 | |
| | | *Scutellospora* spp. | + | / | + | 0 | - | |
| | | *Gigaspora* spp. | 0 | / | 0 | 0 | - | |
| Tomato | *Meloidogyne incognita* | *Glomus coronatum* | 0 | 0,+ | + | + | - | Diedhiou *et al.*, 2003 |
| Banana | *Radopholus similis* | *Glomus mosseae* | 0 | + | 0 | - | 0 | Elsen *et al.*, 2003 |
| | *Partylenchus coffeae* | | 0 | | + | - | - | |

# 2 General materials and methods

## 2.1 General plant cultivation and growing conditions

Unless stated otherwise; tomato (*Lycopersicon esculentum* Mill.) cultivars King Kong II (Known-You Seed Co., Ltd. Taiwan), Hildares (HILD Samen GmbH, Marbach, Germany) and Tip top (Weigelt & Co., Erfurter Samenzucht) were grown from seeds in trays in commercial peat-based substrate (Fruhstorfer Erde Typ P, Fa. Flormaris, Germany). When plants reached the 4-5 leaf stage they were transplanted into a 3:1 mixture of sterile sand and commercial peat-based substrate mixture for use in the experiments. Plants were watered adequately throughout the experiment period and fertilized weekly with 0, 2 % Wuxal top N (N P K 12-4-6, Aglukon Ltd., Düsseldorf, Germany). Experiments were conducted in a greenhouse with a day-night cycle consisting of 16 h of light at 28°C and eight hours of darkness at 24°C at a relative humidity of 80% and a photosynthetic photon flux density of 600-700 $mmol \cdot m^{-2} \cdot s^{-1}$ .

## 2.2 General harvesting procedures

At each harvest, shoots were cut at soil surface, weighed then oven-dried (48 h, 70°C). Roots were washed free of soil, blotted dry and weighed. For experiments where root length was considered as an interaction parameter, roots were cut 1-2 cm segments and root length was measured with a Comair root scanner.

## 2.3 Arbuscular mycorrhizal fungi (AMF)

Four mycorrhizal isolates were used in this work. Three were obtained from the collection of the Institute of Plant Disease and Plant Protection / Hanover University, and those were: No. 510, No. 49, and No. 139. An additional isolate from a tropical forest in Uganda (obtained from Dr. H. Baltruschat, Justus-Liebig University, Gießen), No. 36 was also used.

### 2.3.1 Multiplication of AMF inoculum

The mycorrhizal inoculum was multiplied on Marigold (*Tagetes erecta*) cv. Sonnenschein (Carl- Sperling & Co., Lüneburg) for 12 weeks under greenhouse conditions on expanded clay (LECA®) as described by Dehne and Backhaus (1986).

### 2.3.2 AMF identification

Grouping AMF isolates as taxonomic units is possible using morphological criteria of spores and other fungal structures. Based on morphological criteria the isolates used were identified in the collection as follows: No. 510 as *Glomus intraradices*, No. 49 registered in the European Bank of Glomales as BEG148 and identified as *G. intraradices*, No. 139 as *G. etunicatum* and No. 36 as *G. versiforme*.

However, identification is an important requisite and species determination based on morphology criteria requires considerable experience of spore morphology of a wide range of AMF. Especially that in some cases, the criteria considered in species determination (such as spores size and colour) are continuous rather than discrete, thus, isolates may not be easily distinguished. The most recent approaches focus on the use of Polymerase chain Reaction (PCR) combined with the group specific primers.

PCR is an *in vitro* technique enabling chemical amplification of DNA, it has opened the possibility to analyse organisms at the nucleic acid level even when only small amount of nucleic acid can be obtained, as in the case of AMF. Ribosomal genes have various characters that make them a target choice for phylogenetic and taxonomic studies; they are multicopy genes tandemly organized in the genome. In eukaryotes, each ribosomal gene encodes for three subunits: small subunit, 5.8 subunit, and the large subunit. The three subunits are conserved coding regions unlike the internal transcribed space separating them, which mutate frequently. A protocol to amplify the 5´ end of the large ribosomal unit of Glomeromycota, using AMF spore as starting material was adopted (van Tuinen *et al.*, 1998; Turnau *et al*., 2001).

#### 2.3.2.1 *Preparation of the nucleic acids*

AMF spores (isolates 510 and 36) were collected by wet sieving and rinsed well with distilled water. 5-10 spores were picked under a dissection microscope and transferred into 1.5 ml Eppendorf tubes containing 10 µl distilled water. The spores were crushed within the Eppendorf tubes with a sterile pipette tip. 30 µl 100mM Tris/HCL (pH 8.0) and 10 µl of 20% Chelex 100 were added immediately to the crushed spores. The eppendorfs were incubated at 95°C for 5 minutes, and then cooled on ice. The suspension was then cleared by centrifugation for 1 minute at 10 000 *g*, and the pellet

was discarded, the obtained supernatant was directly used as template for PCR amplification.

#### 2.3.2.2 *Preparation of the PCR reaction*

Each reaction mixture contained:

5µl template DNA,
2µl 10 PCR reaction buffer containing 15 mM $MgCl_2$,
2µl dNTP stock solution 1 mM,
1µl primer 1 (LR1, tab.2.1) stock solution 10 µM,
1µl primer 2 (NDL2, tab. 2.1) stock solution 10 µM;

Each tube was filled to a final volume of 50µl with double distilled water and 1U *Taq* DNA polymerase (stock solution 5U/µl) was added for each reaction tube. Negative control reaction without template DNA.

PCR cycles were performed as follows:

Initial denaturation: at 95°C for 3 min.
Denaturation for the remaining cycles: at 93°C for 45 s
Annealing: at 62°C for 45 s
Extension: at 72°C for 45 s
A final extension of 5 min. was performed at the end of the cycles. In total 33 cycles were performed.

The PCR products were checked by separating 5µl aliquots of the amplification products on a 1.2% agarose gel (Invitrogen, Karlsruhe, Germany) and visualised by ethidium bromide staining.

#### 2.3.2.3 *Nested PCR reaction*

To increase the specificity of the amplification; a second PCR reaction was performed using a 1:10 dilution of the first amplification product and an internal primer (FL2, tab. 2.1). The amplification reaction was performed as mentioned above.

Table 2.1: List of the primers used for AMF species determination and their sequences.

| Primer | Sequence |
|---|---|
| LR1 | 5′-GCA TAT CAA TAA GCG GAG GA- 3′ |
| NDL2 | 5′-TGG TCC GTG TTT CAA GAC G-3′ |
| FL2 | 5′- TGG TCC GTG TTT GAC G-3′ |

#### 2.3.2.4 *Cloning and transformation*

PCR fragments were cloned into pCR2.1-TOPO vector (Invitrogen, Karlsruhe, Germany) according to the instructions of the manufacturer. 4µl aliquot of the PCR product, 1µl salt solution and 1µl of the vector solution were mixed and incubated for 5 min. at room temperature. This ligation solution was placed on ice to be used for transformation of the bacteria.

The -80°C stored component cells were thawed on ice, 2µl of the ligation solution was added and incubated further 30 min. on ice, followed by 30 sec. heat shock at 42°C. The tubes were then cooled on ice for 2 min. and 250µl S.O.C. medium was added and the cells were shaken (225 rpm) in an incubator at 37°C for 1 hour. The cells were then spread on LB plates for white/blue screening and incubated overnight at 37°C.

For analysing positive clones, 8 white colonies were picked and cultured overnight in LB medium containing 50µg/ml ampicillin. Plasmid DNAs were prepared with NucleoSpin® Plasmid kit following the manufacturer's instruction. Sequences obtained were analysed and expressed in a neighbour-joining tree (Fig. 2.1)

### 2.3.3 MPN test

The number of infective AM propagules per $cm^3$ of inoculum was determined by the most probable number (MPN) technique, using pre-germinated marigold seedlings as test plants. Dilutions with sterilized sand (1/10, 1/100 and 1/1000) were used in the test. The diluted samples were transferred to multi-well trays. The marigold seedlings were then planted in each well. There were five replicates per dilution. The plants were watered moderately. After 30 days all root systems were harvested separately. The MPN of AM fungal propagules for each inoculum was calculated.

### 2.3.4 AMF inoculation

For inoculation, the substrate of the related treatments was mixed thoroughly with the AMF inoculum. Initial AMF inoculation levels are described later in the experiments designs.

### 2.3.5 Assessment of AMF variables: Staining and colonization

To visualise AMF colonization, roots were cleared by immersion in 10% KOH (w/v) at room temperature overnight, rinsed with tap water then stained with 2% black ink (Pelikan Co., Hannover)/ household vinegar (5% acetic acid) solution (Vierheilig *et al.*, 1998, modified). The degree of AMF colonization in the root systems was determined microscopically using 20 1-cm root segments. Mycorrhization was expressed as percentage and calculated as follows:

$$\% \text{ Colonization} = \frac{\text{Root pieces colonized by the fungus}}{20} \times 100$$

Intensity of colonization was rated on a scale from 0-3 with 0 = no colonization and 3 = completely colonized by all fungal particles (Backhaus, 1984).

## 2.4 Nematodes

The source of nematode inocula was a population of *M. incognita* (race 3) maintained as a stock culture on tomato *Lycopersicon esculentum* cv. Hildares growing in sterile sand.

### 2.4.1 Nematode inocula

Nematode eggs were extracted from heavily galled roots as described by Hussey and Barker (1973). Roots were washed, cut into 1-2cm segments and macerated in a blender for 20 seconds. The macerate was filled into a flask containing 500ml of 1.25% NaOCl solution and shaken for 3 minutes to liberate eggs from the gelatinous matrix. Eggs were separated from plant debris by passing the egg suspension successively through sieves of 200µm, 100µm and 25µm mesh size. To remove excess chlorine, the eggs from the 25µm sieve were washed several times with tap water. The eggs were passed again through a sieve combination of 45µm and 20µm mesh sizes. Eggs from the 20µm sieve were then collected in tap water. For inoculation aliquots of egg suspension were mixed with the substrate.

When second-stage juveniles (J2) were required as inoculum, eggs were collected as mentioned above. The resulting egg suspension was diluted with tap water and agitated for three days at 24°C to induce juvenile hatching. Active juveniles were separated from eggs using Oostenbrick trays method (Oostenbrink 1960). The juveniles were suspended in tap water and inoculated in holes made around the stem base.

### 2.4.2 Assessment of nematode variables

#### 2.4.2.1 Nematode infection

The nematode gall index was rated on a 0-10 scale with 0 = no galls and 10 = completely galled (Zeck, 1971). Numbers of galls and egg masses induced were counted under a dissecting stereomicroscope. For the counting of eggmasses roots were immersed in aqueous solution of Ploxine B (0.15 g $l^{-1}$) for 15 minutes.

### 2.5 Statistical analyses

Data were analysed according to the standard analysis of variance procedures using SAS (version 8.2, SAS Institute Inc., Cary, NC, USA). To normalize variances for all analyses, the number of galls and eggsacs per gram roots were square root transformed after adding a constant factor of 0.5, mycorrhizal infection frequencies were arcsin transformed and bacterial population counts were transformed to log ($\chi$ + 1). Data were then analysed using the GLM procedure. Treatment means were separated using Tukey's or Duncan's multiple range tests. Statistical differences referred to in the text are significant at $P \leq 0.05$. Non-parametric analysis of variance was run for the data of the histology studies.

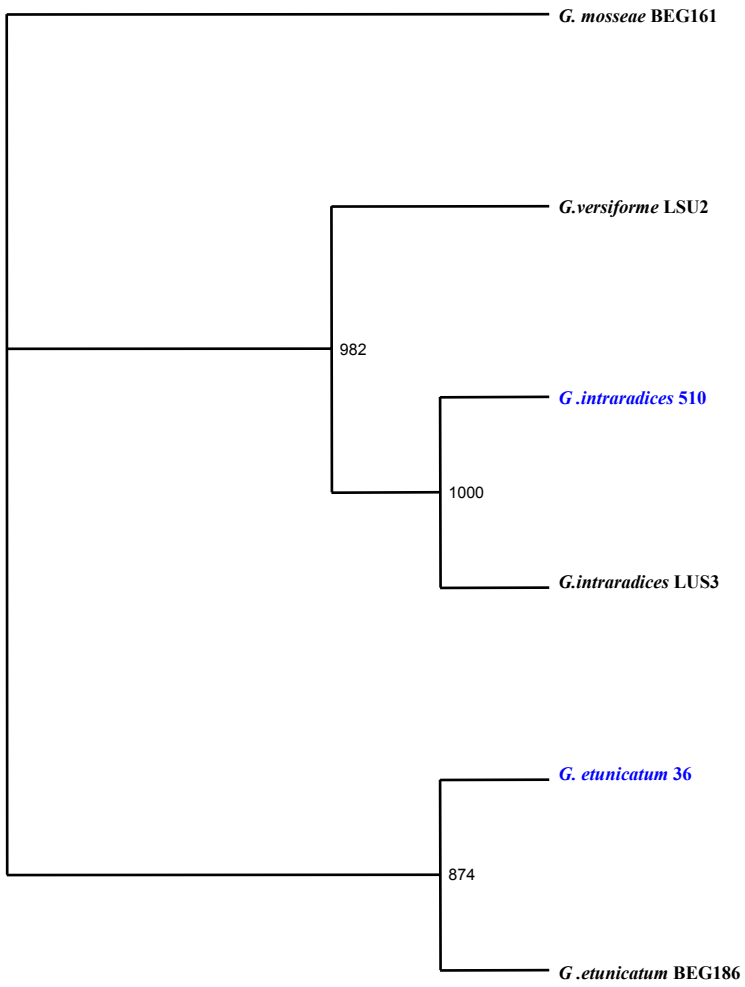


Figure 2.1: Phylogenetic tree obtained from the alignment on 670 base of the Large ribosomal subunit. Percentage bootstrap (out of 1000 trails) is indicated. Sequences obtained in this work are in blue, compared to sequences published in the Gene Bank.

**2.6 Remarks**

The Alignment of the sequences obtained was made on 670 base of the large ribosomal subunit. The sequences were aligned with Clustalw. The neighbourjoining tree was tested with th e bootstrab method. The Results obtained from the molecular identification of the AMF isolates confirms the earlier identification of the AMF isolate 510. This isolate is highly homologous with *Glomus intraradices*. On the other hand, the molecular identification of the AMF isolate 36 reveals it similarity to *Glomus etunicatum* and not to *G. versiforme* as was assumed.

# 3 Study of the interaction aspects between arbuscular mycorrhizal fungi and the root-knot nematode *Meloidogyne incognita* in tomato.

**Abstract**

Nematode-mycorrhiza interactions appear to be highly dependent on the given association of plant cultivar, nematode species, and AMF species or isolate. An isolate of *G. intraradices* (no. 510) reduced gall numbers induced by *M. incognita* in the roots of the tomato cultivar Kingkong II, but had no influence on the number of eggsacs, while *G. etunicatum* (no. 139) reduced both gall and eggsac numbers. In contrast, a tropical isolate of *G. etunicatum* (no. 36) had no influence on galls and eggsacs, while *G. intraradices* (no. 49) exhibited a non consistent effect. All tested mycorrhizal isolates reduced gall size. The sequence of aspects in the interaction between AMF and *M. incognita* has been tested. Differences (between mycorrhizal and non-mycorrhizal treatments or among mycorrhizal treatments themselves) in final number of galls were not attributed to differences in nematode pre-infectional aspects. Root diffusates collected from mycorrhizal and non-mycorrhizal plants did not influence egg hatch of *M. incognita*. Second stage juveniles were less attracted to mycorrhizal plants than non-mycorrhizal plants in a pair-choice assay and were slower in invading roots inoculated with *G. intraradices* (no. 510) in an invasion assay. However, the final number of nematodes invaded mycorrhizal roots was similar as in non-mycorrhizal roots. Nematode suppression by *G. intraradices* (no. 510) is partially attributed to induced resistance mechanisms.

## 3.1 Introduction

Root-knot nematode parasitism of plants is a complex dynamic interaction that involves hatching stimuli, attraction to the host, invasion of the host tissue, feeding site formation, and an active response from the host (Bleve-Zacheo and Melillo, 1997). These aspects are generally categorized into pre- and post-infectional (Thomson Cason *et al.*, 1983; Trudgill, 1992). Pre-infection aspects may occur within the rhizosphere or at the root surface and likely involve signals from roots; thereby influencing egg hatch, attraction toward the roots, and attraction and penetration of the target tissues (Thomson Cason *et al.*, 1983; Trudgill, 1992; Perry, 1997; Zhao *et al.*, 2000). Post-infectional aspects involve physiological processes within the roots which affect: 1) nematode feeding, 2) establishment of feeding sites, 3) nematode development, and 4) reproduction (Trudgill, 1992).

Over many years, research efforts were undertaken to identify factors involved in the different nematode interaction aspects (Viglierchio, 1961; Lownsbery and Viglierchio, 1961; Abou-Setta and Duncan, 1998; Zhao *et al.*, 2000). However, the primary focus was on comparing hosts to non-hosts, susceptible to resistant cultivars, and plants of different host status. The aspects of the nematode/host interaction are very likely to be affected by co-occurring organisms such as arbuscular mycorrhizal fungi. The descriptive model of Hussey and Roncadori (1982) was used as a framework in studies on the interaction of nematodes and mycorrhizal fungi. The model considered mycorrhizal effects on host efficiency (measured in terms of nematode, or egg densities, or nematode development in mycorrhizal plants compared to non-mycorrhizal plants) and host sensitivity (determined in terms of growth or yield suppression), which describes the final outcome of the association. However, the detailed knowledge regarding the precise aspects of interaction between nematodes and AMF can be beneficial in designing efficient integrated pest management (IPM) systems.

### 3.2 Objectives and experimental program

The objectives of the presented group of experiments were to screen for an effective AMF isolate that confer a bioprotective activity against the RKN *M. incognita* and to characterize the influence of AMF on the different nematode pre- and post-infectional aspects. An additional objective was to elucidate possible involved mechanisms.

For differentiation of the full extent interaction processes, independent but complementary experiments were used. The tested interaction aspects were: hatching, attraction, invasion, and development as reflected by gall size and egg laying, as well the development of feeding sites.

#### 3.2.1 Interaction of AMF, RKN and tomato (cv. Kingkong II): Influence of nursery AMF treatments on nematode infection and mycorrhizal colonization extension into AMF-free soil

##### 3.2.1.1 Experimental set-up

The experiment was designed to simulate common techniques of tomato production where seedlings are first produced in pathogen free soil in the nurseries and then transplanted into the infected field soil. Five-days old tomato seedlings were potted in 500 ml plastic pots; the substrate at this stage was thoroughly mixed with 10% AMF inocu-

lum, substrate of the control was mixed with AMF-free expanded clay. Two weeks later –when mycorrhizal colonization ≈ 25%- the plastic pots were substituted with mesh pots and then transplanted in 2 l pots containing the same substrate mixture, inoculated or non-inoculated with 1250 *M. incognita* eggs; according to the treatment (Figure 3.1). Plants were fertilized weekly with 0,2 % Wuxal top N and harvested forty days after being potted. Shoot fresh and dry weights were recorded. Roots in the central compartment were separated, washed, and weighed independently from those grown in the outer compartment. Number of galls (3 one-gram counts per replicate) and diameters of galls (15 measurements per replicate) induced by *M. incognita* were evaluated using a stereomicroscope. The final numbers of galls per root system were calculated (average of galls per gram × root weight). AMF colonization density and intensity were rated using light microscope (see: 2.3.5).

This experiment was conducted three times at different time frames during the research work (Experiment I: Dec. 2002 –Feb. 2003, Experiment II: Jun.-Aug. 2003, Experiment III: Jan.-Mar. 2004), the results of each are compared later to its relative control. Experiment III was conducted to confirm the results obtained earlier, to compare further parameters, and to test an AMF isolate that was multiplied later.

Treatments of the different experiments were as follows:

**Experiment I**:

1. non-inoculated (C),
2. inoculated with *M. incognita* (Mi),
3. inoculated with *G. intraradices* 510 (Gi 510),
4. inoculated with *G. etunicatum* 36 (Ge 36),
5. inoculated with *M. incognita* and *G. intraradices* 510 (Gi 510 + Mi),
6. inoculated with *M. incognita* and *G. etunicatum* 36 (Ge 36 + Mi).

**Experiment II:**

1. non- inoculated (C);
2. inoculated with *M. incognita* (Mi);
3. inoculated with *G. intraradices* 49 (Gi 49);
4. inoculated with *M. incognita* and *G. intraradices* 49 (Gi 49 + Mi).

**Experiment III:**

1. non- inoculated (C),
2. inoculated with *M. incognita* (Mi);
3. inoculated with *M. incognita* and *G. intraradices* 510 (Gi 510 + Mi);
4. inoculated with *M. incognita* and *G. etunicatum* 36 (Ge 36 + Mi);
5. inoculated with *M. incognita* and *G. intraradices* 49 (Gi 49 + Mi);
6. inoculated with *M. incognita* and *G. etunicatum* 139 (Ge 139 + Mi);
7. inoculated with negative inoculum i.e. produced following the same procedures of AMF inoculum production but without the mycorrhizal fungus so that it contains the micro-organisms that are not specifically associated with the AMF (Ne + Mi).

The treatments were laid out in a completely randomised design and each consisted of 6 replicates.

Based on the results obtained from experiments I and II (data obtained until August 2003), the AMF isolate conferring bioprotection against *M. incognita* was further tested. The interaction was tested in a stepwise manner to verify the event that is most affected by the mycorrhizal symbiosis.

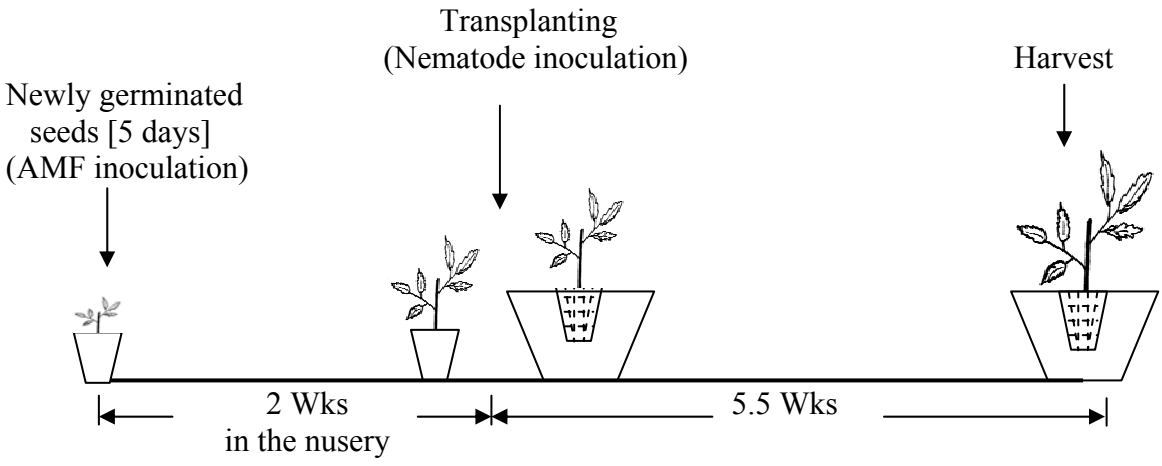


Figure 3.1: Schematic overview of the time frames used in the set-ups of the mesh pot experiments.

### 3.2.1.2 Results

Table 3.1 shows the data obtained in the experiments studying the influence of nursery AMF treatments on nematode control (experiments I and II). Neither nematode infection nor mycorrhizal colonization had an effect on shoot weight. Nematode infection increased root weight. Generally, the tested AMF isolates did not induce different growth responses. Inoculation of *G. intraradices* 510 reduced the number of galls induced by *M. incognita*; however, the reduction was not significant. On the other hand, *G. etunicatum* 36 did not influence the number of nematode galls. In contrast to gall numbers, gall size was clearly reduced by all mycorrhizal treatments (Figure 3.2, a-b). Mycorrhizal infection by all AMF isolates ranged between 83 % and 95 % and did not significantly differ between the inner (inoculated) root periphery and the outer one. Nematode infection did not influence mycorrhizal colonization (Table 3.1).

In experiment III (Table 3.2), the results obtained exhibited no plant response in terms of growth parameters (shoot fresh weight, shoot dry weight, root weight, and root length) to any of the treatments. The numbers of galls (per gram roots) induced by *M. incognita* were significantly reduced in the inner and outer compartments of *G. intraradices* 510 and *G. etunicatum* 139 treatments (Table 3.3) and per plant (Figure 3.3), whereas, the reduction of gall numbers by *G. etunicatum* 36 was not significant. The influence of *G. intraradices* 49 was restricted to the inner root periphery (Table 3.3), and resulted in a reduction in the total number of galls per root system (Figure 3.3). Interestingly, the negative (control) inoculum reduced the total number of galls; this reduction was statistically similar to *G. etunicatum* 36 and *G. intraradices* 49 (Figure 3.3). Except for the treatment *G. etunicatum* 139 there was no difference in the distribution of galls between the inner and the outer root peripheries (Figure 3.4). Fewer galls were induced in the inner root periphery of *G. etunicatum* 139 compared to the outer root part. *G. etunicatum* 139 was the only treatment that yielded significantly less eggsacs (Table 3.3, Figure 3.3). The density of mycorrhizal infection of all AMF ranged between 85 % and 90 % and did not differ between the inner and outer root parts (Table 3.3). As in the results of set I and II, nematodes had no effect on mycorrhizal density. Figure 3.2 (c) exhibits clearly that even when mycorrhizal colonization did not affect the number of galls, it reduced gall size. This influence was general for all isolates tested.

Table 3.1: Plant, nematode and mycorrhizal interaction parameters in the experiments studying the influence of nursery AMF treatments on nematode control; experiment I and II. Root fresh weight (R FWT), shoot fresh weight (Sh FWT), frequency of mycorrhizal colonization (MF, in % colonized root pieces of 1 cm length), and numbers of galls per g in the inner and the outer root peripheries (IR and OR respectively) and per plant (± SE).

| | Treatment | R FWT [g] | | Sh FWT [g] | | Galls/g - IR | | Galls/g – OR | | MF - IR [%] | MF - OR [%] | Galls/plant |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment I | ***G. intraradices* 510** | 38.1 ± 1.0 | **abc** | 154.3 ± 9.9 | **bc** | | | | | 95.8 ± 2.3 | 95.8 ± 2.3 | |
| | ***G. intraradices* 510 + *M. incognita*** | 45.4 ± 2.0 | **ab** | 171.0 ± 6.3 | **ab** | 32.1 ±5.3 | **b** | 42.3 ±8.4 | **b** | 92.5 ± 2.1 | 91.7 ± 3.3 | 680.8 ± 113.0 |
| | ***G. etunicatum* 36** | 32.9 ± 2.2 | **c** | 150.4 ± 5.7 | **bc** | | | | | 95.8 ± 1.5 | 91.7 ± 2.7 | |
| | ***G. etunicatum* 36 + *M. incognita*** | 35.9 ± 4.1 | **bc** | 157.8 ± 5.9 | **bc** | 62.9 ±9.4 | **a** | 83.3 ± 1.7 | **a** | 88.3 ± 3.3 | 83.3 ± 3.5 | 987.5 ± 128.4 |
| | **Control** | 31.4 ± 1.4 | **c** | 161.8 ± 7.8 | **ab** | | | | | | | |
| | ***M. incognita*** | 47.6 ± 2.7 | **a** | 199.3±14.7 | **a** | 50.3 ± 10.0 | **ab** | 63.8 ± 5.7 | **ab** | | | 992.9 ± 103.5 |
| Experiment II | ***G. intraradices* 49** | 50.5 ± 2.5 | **ab** | 158.0 ± 3.2 | | | | | | 95.0 ± 1.7 | 95.8 ± 2.2 | |
| | ***G. intraradices* 49 + *M. incognita*** | 54.5 ± 3.2 | **a** | 143.9 ± 4.9 | | 64.2 ±2.7 | **a** | 60.2 ± 6.3 | | 92.5 ± 2.1 | 98.3 ± 1.7 | 934.1 ± 27.1 |
| | **Control** | 43.6 ± 2.4 | **b** | 151.6 ± 5.6 | | | | | | | | |
| | ***M. incognita*** | 51.7± 2.2 | **ab** | 157.9 ± 3.5 | | 42.2 ±4.3 | **b** | 77.9 ± 6.8 | | | | 962.3 ± 26.9 |

Within the sets values in the same column followed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

Table 3.2: Plant growth parameters in the experiment studying the influence of nursery AMF treatments on nematode control and mycorrhizal extension; experiment III. Shoot fresh weight (Sh FWT), shoot dry weight (Sh DWT), root fresh weight (R FWT), root length (R L) of the inner and the outer root peripheries (IR and OR respectively) of plants (±SE), n.= 6.

| Treatment | Sh FWT [g] | Sh DWT [g] | R FWT [g] | | | R L [m] | |
|---|---|---|---|---|---|---|---|
| | | | IR | OR | Total | IR | OR |
| **Control** | 95.6 ± 1.8 | 17.8 ± 0.4 | 14.2 ± 0.6 | 10.5 ± 0.7 | 24.7 ± 1.1 | 51.2 ± 2.6 | 62.8 ± 1.9 |
| ***M. incognita*** | 88.2 ± 5.8 | 17.1 ± 0.9 | 15.0 ± 1.2 | 10.5 ± 1.0 | 25.5 ± 1.9 | 53.2 ± 3.5 | 58.4 ± 6.1 |
| ***G. intraradices* 510 + *M. incognita*** | 89.8 ± 2.3 | 17.9 ± 1.0 | 13.5 ± 1.0 | 9.8 ± 0.7 | 23.2 ± 1.5 | 50.5 ± 1.3 | 57.8 ± 2.4 |
| ***G .etunicatum* 36 + *M. incognita*** | 103.0 ± 7.1 | 18.8 ± 0.8 | 13.6 ± 1.0 | 9.9 ± 0.6 | 23.5 ± 1.6 | 58.7 ± 3.1 | 54.6 ± 3.2 |
| ***G. intraradices* 49 + *M. incognita*** | 92.2 ± 1.4 | 18.8 ± 1.3 | 14.3 ± 0.9 | 11.2 ± 1.4 | 25.5 ± 1.5 | 55.7 ± 1.6 | 55.5 ± 4.9 |
| ***G. etunicatum* 139 + *M. incognita*** | 83.9 ± 3.4 | 17.2 ± 0.8 | 16.6 ± 1.2 | 9.3 ± 0.8 | 25.9 ± 2.0 | 56.7 ± 2.5 | 56.9 ± 7.9 |
| **Negative inoculum + *M. incognita*** | 103.0 ± 6.9 | 19.4 ± 1.2 | 13.0 ± 0.7 | 10.2 ± 0.9 | 23.3 ± 1.5 | 48.9 ± 2.2 | 61.2 ± 2.9 |

Table 3.3: Nematode and mycorrhizal parameters in the experiment studying the influence of nursery AMF treatments on nematode control and mycorrhizal extension; experiment III. Numbers of galls per g, numbers of eggsacs per g, frequency of mycorrhizal colonization (MF, in % colonized root pieces of 1 cm length), in the inner and the outer root peripheries (IR and OR respectively), and number of galls or eggsacs per plant (± SE), n = 6.

| Treatment | Galls/ g | | | | Eggsacs/g | | | | MF [%] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | IR | | OR | | IR | | OR | | IR | OR |
| ***M. incognita*** | 48.9 ± 1.8 | **a** | 41.2 ± 5.2 | **a** | 7.1 ± 1.1 | **a** | 10.1 ± 1.4 | **a** | | |
| ***G. intraradices* 510 + *M. incognita*** | 23.2 ± 2.1 | **b** | 22.4 ± 4.6 | **b** | 5.3 ± 0.9 | **a** | 5.4 ± 0.4 | **b** | 88.3 ± 4.4 | 90.83 ± 3.52 |
| ***G.etunicatum* 36 + *M. incognita*** | 46.6 ± 2.4 | **a** | 28.6 ± 3.2 | **ab** | 6.6 ± 1.1 | **a** | 7.2 ± 1.6 | **ab** | 86.6 ± 3.3 | 85.83 ± 3.27 |
| ***G. intraradices* 49 + *M. incognita*** | 29.6 ± 2.7 | **b** | 33.9 ± 7.3 | **ab** | 4.7 ± 0.8 | **a** | 9.5 ± 1.9 | **a** | 90.0 ± 2.6 | 85.83 ± 3.96 |
| ***G.etunicatum* 139 + *M. incognita*** | 3.2 ± 0.4 | **c** | 11.5 ± 1.2 | **c** | 0.3 ± 0.2 | **b** | 4.8 ± 0.7 | **b** | 89.2 ± 3.0 | 85.83 ± 1.54 |
| **Negative inoculum + *M. incognita*** | 34.1 ± 7.1 | **ab** | 29.3 ± 3.0 | **ab** | 5.0 ± 1.6 | **a** | 9.6 ± 1.6 | **a** | | |

Values in the same column followed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

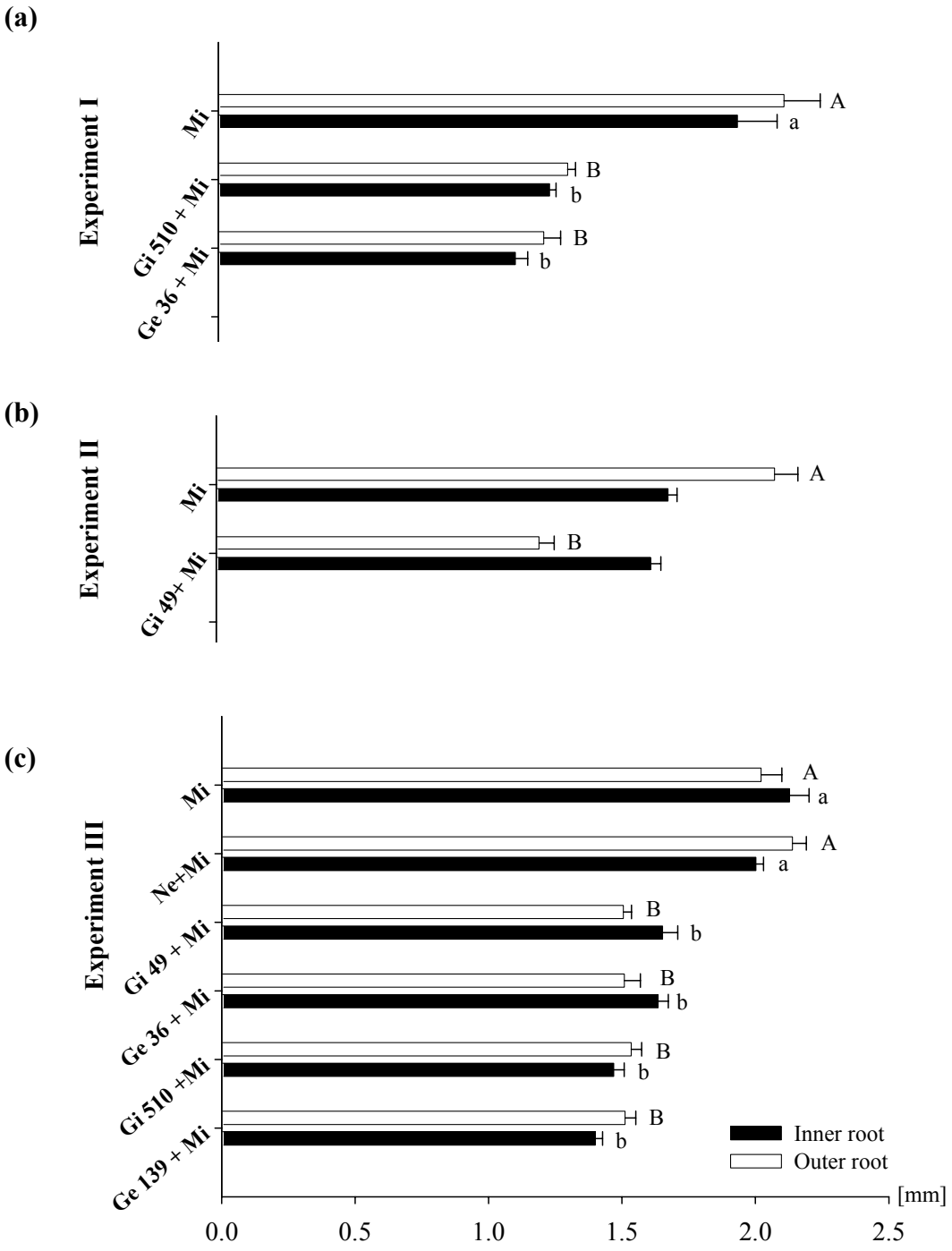


Figure 3.2: Diameter of galls (± SE), induced by *M. incognita* (Mi) in the inner and outer root parts of mycorrhizal (*G. intraradices* isolates 49 and 510, *G. etunicatum* isolates 36 and 139) or non-mycorrhizal plants (Ne) in the experiments studying the influence of nursery AMF treatments on nematode control and mycorrhizal extension; experiments III. I (a), II (b), and III (c). Bars followed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

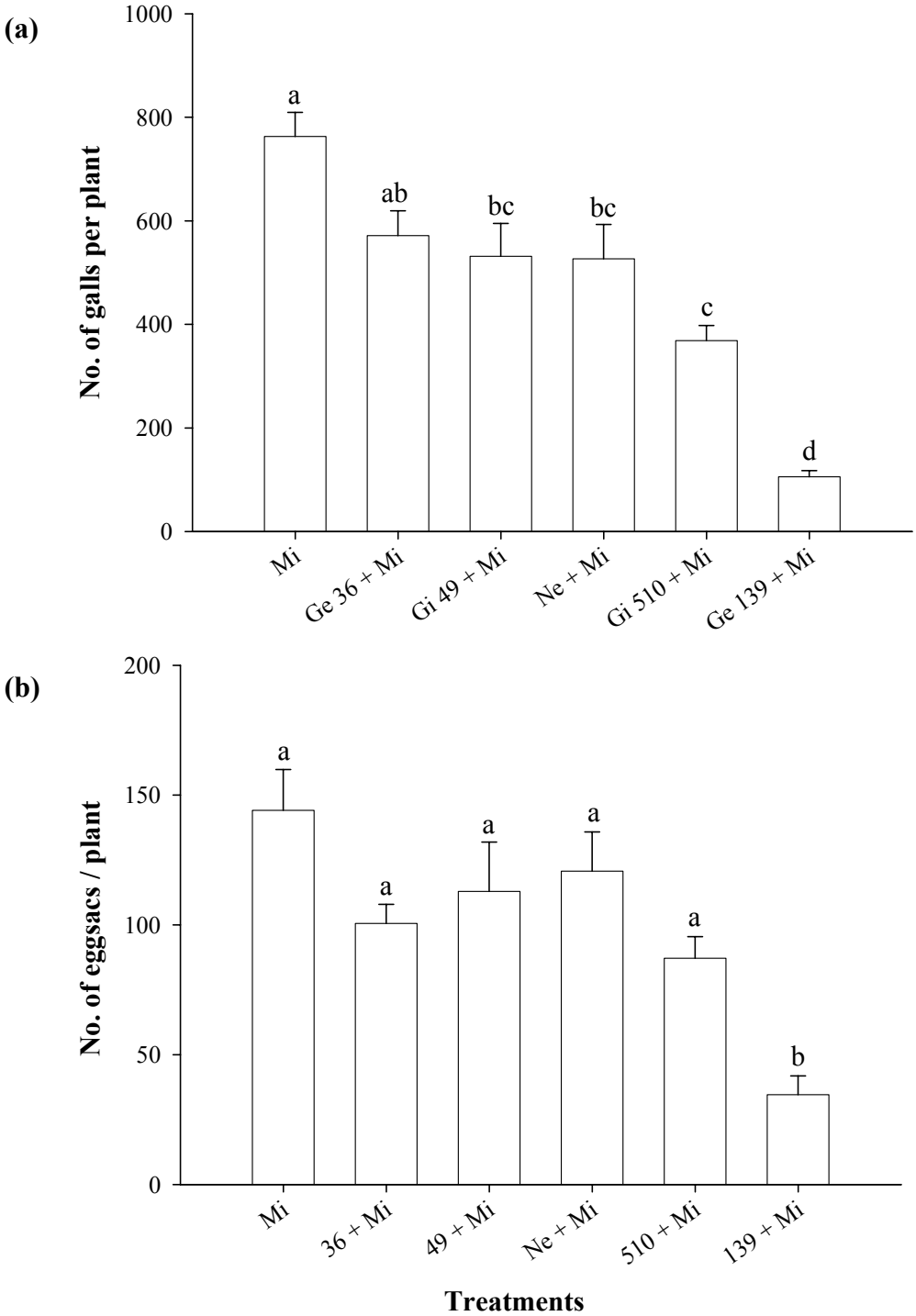


Figure 3.3: Number of galls (a) and eggsacs (b) (± SE), induced by *M. incognita* per plant in the experiment studying the influence of nursery AMF treatments on nematode control and mycorrhizal extension; experiment III. Treatments were inoculated with, *G. intraradices* 510 (Gi 510), or 49 (Gi 49), *G. etunicatum* 36 (Ge 36) or 139 (Ge 139), inoculated with negative inoculum (Ne), or non-mycorrhizal (Mi). Bars headed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

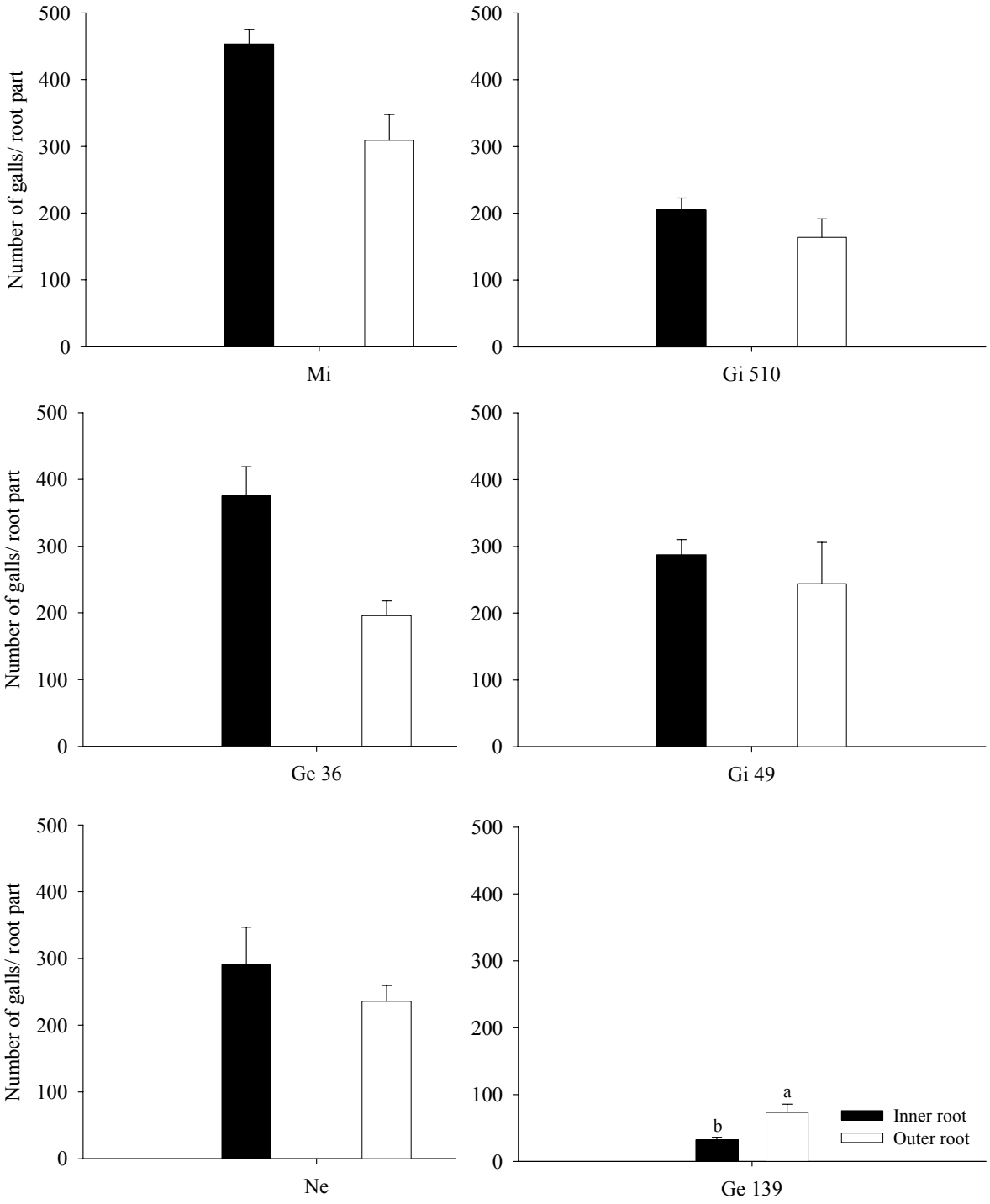


Figure 3.4: Distribution of galls (± SE), induced by *M. incognita*, in the inner and outer root peripheries of the different treatments in experiment studying influence of nursery AMF treatment on nematode control and mycorrhizal colonization extension experiment III. Treatments were inoculated with, *G. intraradices* 510 (Gi 510), or 49 (Gi 49), *G. etunicatum* 36 (Ge 36) or 139 (Ge 139), inoculated with negative inoculum (Ne), or non-mycorrhizal (Mi). Bars headed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

### 3.2.1.3 Discussion

It is generally observed that increased resistance or tolerance of plants to pathogens is associated with a well established mycorrhizal infection (Dehne, 1982). Inoculation of the plants in the nursery with AMF allows sufficient time to establish the symbiosis before transplanting and prior to pathogen exposure (Calvet *et al.*, 2001; Talavera *et al.*, 2001). The presented group of experiments was designed to meet these observations and to simulate common techniques of tomato production; where seedlings are first produced in pathogen free soil in the nurseries and are then transplanted into the field. The experimental set-up permitted also to differentiate the development of AM and nematode infection in the different root peripheries.

Under field conditions beneficial effects of AM on nematode-infested plants can often be due to a better plant nutrition of mycorrhizal plants that are then more vigorous and able to compensate. In spite of the intense mycorrhizal colonization, growth benefits due to AMF inoculation were not observed. In literature, the lack of a growth response to AM in greenhouse tomato plants under experimental conditions was often observed (Daft and Nicholson, 1972; Bagyaraj *et al.*, 1979; Thomson Cason *et al.*, 1983; Diedhiou *et al.*, 2003). Moreover, it is well established that this mycorrhizal effect is highly dependent on the involved plant and fungal species (Plenchette *et al.*, 1983). In the presented study, beneficial nutritional effects of AM and detrimental influence of nematodes are likely excluded by the good growth conditions in the greenhouse, favouring rapid growth and nematode tolerance (Netscher and Sikora, 1990). The tendency of higher root weight in the nematode treatments observed in some of the presented experiments (mesh pot- experiments I and II) can be attributed to the hypertrophic root tissue of the *M. incognita* galls.

Mycorrhizal infection by all AMF isolates did not significantly differ between the inner (inoculated) and the outer root peripheries and was not influenced by nematode infection. Nematodes may physically disrupt root tissues and cause physiological alterations that impeding AMF colonisation (Brussaard *et al.*, 2001). However, in the presented experiment the mycorrhization was rather intense and does not appear to have been affected by nematode infection, the results corroborate with those obtained by Jaizme-Vega *et al.* (1997), where *M. incognita* did not affect the colonization of *G. mosseae* in banana.

The significantly lower gall incidence in plants inoculated with *G. intraradices* 510 and *G. etunicatum* 139 (experiments I and III) confirm reports on the ability of AMF to suppress phytonematodes (Jaizme-Vega *et al.*, 1997; Parvatha Reddy *et al.*, 1998; Elsen *et al.*, 2001; Diedhiou *et al.*, 2003; Elsen *et al.*, 2003). *G. etunicatum* 139 was the most effective in reducing both nematode infection parameters; no. of galls and no. of eggsacs, followed by *G. intraradices*. Similar influence on gall indices of *M. incognita* by an isolate of *G. intraradices* on tomato was reported by Suresh *et al.* (1985). On the other hand, the results obtained from other AMF treatments (*G. etunicatum* 36 and *G. intraradices* 49; experiments I, II and III) agree with other studies where no nematode suppression by AMF was conferred (Thomson Cason *et al.*, 1983; Vaast *et al.*, 1998). The numbers of galls represent the number of nematodes invaded the root and developed, while numbers of eggsacs represent the egg-laying females thus the rate of development. The lower galls indices in the *G. etunicatum* 139 and *G. intraradices* 510 treatments suggest reduced hatching, reduced invasion, or – when hatching and/or invasion are not involved- the failure of more of nematodes to develop due to post infectional factors. Although mycorrhization by *G. intraradices* 510 negatively affected the number of galls, it seems that females were more advanced in development in this treatment as it yielded similar number of eggsacs compared to the control. The results provide no evidence of a relationship between the treatments and nematode reproduction since the egg output was not evaluated.

The differences in efficacy of the AMF isolates to suppress nematode infection could not be explained by differences in their ability to improve plant vigour and nutrient uptake and thus enhancing plant tolerance; neither could it be attributed to difference in their ability to colonize the roots as mycorrhization did not differ among the AMF treatments. A comparable lack of relation between infectivity and effectivity of AMF in interaction with nematodes was reported before (Elsen *et al.*, 2003). Different mycorrhizal colonization levels would reflect different competition abilities of the isolates. Competition for infection sites is one of the mechanisms by which AMF control root pathogens (Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004). It is obvious that the ability of AMF isolates to prevent gall formation is not related to the ability to colonize roots, suggesting that, competition –if actually involved- is not the only mechanism. Tylka *et al.* (1991) and Elsen *et al.* (2001) suggested that certain AMF iso-

lates are capable to induce a systemic response in plants that would be antagonistic to the development of nematodes.

Among the other mechanisms reported to be involved in pathogens suppression by AMF, are the morphological changes in the root system. Such changes are likely to affect the interaction, particularly nematode-infection. Root system parameters such as root weight and length and number of axes may influence invasion (Cook *et al.*, 1999; Ehwaeti *et al*., 1999). In this regard, no differences among the mycorrhizal treatments or in comparison to the control were observed in terms of root weight and length. However, whether this reflects also similarity in root branching was not evaluated. The most frequent consequence of AM colonization is an increase in branching (Azcón-Aguilar and Barea, 1996). A more branched root system offer more infection sites for juveniles' penetration. More detailed examination for anatomical changes in roots of the different treatments is required to reveal the influence of different AMF isolates on root branching.

Except for *G. etunicatum* 139 treatment, no differences were observed in gall incidence in the two root peripheries. Sikora (1978) demonstrated that the majority of galls induced by *M. incognita* on tomato were negatively correlated with the colonization levels of *G. mosseae* in the root system which was lower in the outer periphery of the root compared to the internal regions. The results obtained here do not exhibit any difference in the mycorrhizal colonisation between the two peripheries – when quantified at the end of the experiment. However, it could be that AMF isolates effectively suppressing nematodes are faster colonizers, which requires evaluation of the mycorrhizal colonization at different phases of the experiment.

In contrast to the differences among AMF isolates on the numbers of galls and eggsacs, all AMF treatments seem to have negatively affected nematode development, resulting in the smaller gall size in all mycorrhizal treatments. Smaller galls under the experimental conditions used indicate younger age of the galls or suppressed development. Direct competition between AMF and endoparasitic nematodes for space inside roots may result in a suppression of nematode development. Moreover, the growth of both endophytes depends on host photosynthates, so competition for carbon compounds could be a cause for smaller nematode galls in mycorrhizal plants. Generally it would be as-

sumed that the smaller the galls the smaller and the less developed the adult inside the gall, growth rate and size of the adults are influenced by food supply and host (Ferris *et al.*, 1984; Hillocks *et al*., 1995; Atkinson *et al.* 1996), which in turn affects the number of generations per growing season and hence the size of nematode population attacking the next crop (Madulu and Trudgill, 1994; Mcloed *et al*., 2001). If smaller galls are simply younger, then smaller gall size in mycorrhizal plants is then due to differences in pre-infectional aspects such as delayed hatching, less attraction to the target tissues and/ or delayed penetration. Synchronization of juveniles' penetration is necessary to determine the factors involved.

### 3.2.2 Influence of root diffusates from mycorrhizal and non-mycorrhizal plants on nematode hatch

In the experiments regarding influence of nursery AMF treatments on nematode infection and mycorrhizal colonization extension into AMF-free soil, some treatments resulted in reduced gall numbers. This reduction could be in principle due to reduced egg hatching making less nematodes attack the roots. To investigate this hypothesis root exudates of the different mycorrhizal treatments were tested in an *in vitro* hatching experiment.

#### 3.2.2.1 Experimental set-up

Tomato seedlings (cv. King Kong II) were transplanted into 600 ml plastic pots and grown for four weeks with and without AMF. Plants were watered daily near to field capacity. In order to obtain root diffusates watering was stopped for two days and root diffusates were then leached with 250 ml water per pot. The crude exudates from five pots in each treatment were pooled, filtered and stored in plastic bottles (Gaur *et al.*, 2000) at 4°C. After collection of root exudates, the root fresh weight was determined and mycorrhizal colonization was assessed. The ratio of fresh weight/root diffusates solution (w/v) was similar in all treatments; therefore, standardization of root exudates concentration was not necessary.

Sterile tissue culture multi-well plates (Greiner Bio-One GmbH, Germany) were used for the hatching test. Each treatment was conducted in a separate plate, five wells in each plate were used; each represented a replicate. The root diffusates used were collected in the first experiment from three treatments; tomato plants inoculated with: the AMF *G. intraradices* 510, *G. etunicatum* 36 and from non-inoculated control plants (Experiment I). Hatching in distilled water served as a control. Diffusates from plants colonized by the AM fungus *G. intraradices* 49 were tested in a second experiment compared with an independent control and water (Experiment II).

Galls of approximately equal sizes, each with one egg-mass, obtained from tomato plants 4 weeks after inoculation with freshly hatched juveniles were used. Three galls were placed in each test well; 1 ml of the root exudates solution was added. Juveniles' counts and replacement of the exudates from stock solution were made at 48 h intervals for 14 days. The plates were incubated in dark at 27 ±1 °C. At the end of the test, the

galls in each test well were immersed in 5ml of a 20% solution of 5.25% sodium hypochlorite (Clorox®) for 15 minutes then vortexed for 1 minute to liberate the remaining eggs from the gelatinous matrix. The percentage hatch was determined as the ratio of the cumulative number of juveniles hatched in the solution to the total number of hatched juveniles plus the eggs remaining in the egg mass.

#### 3.2.2.2 Results

The hatching response by *M. incognita* to tomato root exudates did not differ among diffusates collected from plant colonized by different AMF. Hatching response to root diffusates from AMF plants was similar to that of root diffusates collected from control plants and similar to hatching rate recorded in water in both experimental sets (Table 3.4).

Table 3.4: Cumulative percentage of *M. incognita* hatch (± SE) in root exudates from tomato seedlings colonized by different mycorrhizal fungi and compared to root exudates from non-mycorrhizal (control) plants and water, n=5.

| | Treatments | Days exposed to root exudates | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| Experiment I | **Control** | 5.6 ±0.8 | 18.6 ±2.0 | 29.1 ±3.7 | 45.1 ±5.5 | 52.7 ±5.7 | 66.4 ±5.9 | 77.8 ±4.3 |
| | ***G. intraradices* 510** | 4.5 ±1.2 | 17.6 ±2.2 | 26.9 ±2.9 | 43.1 ±3.5 | 52.1 ±3.4 | 66.4 ±2.7 | 79.3 ±1.9 |
| | ***G. etunicatum* 36** | 7.7 ±1.3 | 21.1 ±2.9 | 34.1 ±4.0 | 46.3 ±4.1 | 57.4 ±4.2 | 71.3 ±3.4 | 82.0 ±1.2 |
| | **Water** | 8.4 ±2.4 | 17.6 ±3.6 | 29.4 ±2.9 | 42.2 ±2.6 | 54.5 ±2.1 | 70.3 ±2.2 | 82.5 ±2.0 |
| Experiment II | **Control** | 4.4 ±0.7 | 16.9 ±1.4 | 31.8 ±1.8 | 49.4 ±2.2 | 62.5 ±2.2 | 78.0 ±1.4 | 92.0 ±0.8 |
| | ***G. intraradices* 49** | 4.0 ±1.1 | 16.1 ±2.3 | 28.8 ±2.9 | 46.3 ±2.2 | 59.2 ±2.4 | 74.8 ±2.8 | 90.4 ±1.5 |
| | **Water** | 6.9 ±1.0 | 18.3 ±1.9 | 30.2 ±1.2 | 42.8 ±1.1 | 58.0 ±2.0 | 75.8 ±1.5 | 90.3 ±0.5 |

#### 3.2.2.3 Discussion

The first recognition event in the interaction between host and nematode is the possible influence of the plant on egg hatching. However, in contrast to some species of cyst nematodes, where stimulation by host root diffusates is required for substantial hatch, most species of *Meloidogyne* hatch in water once the development of J2 has completed; host root diffusates are not required for substantial hatch, but may enhance the rate of hatch (Perry, 1997; Gaur *et al.*, 2000; Zhao *et al.*, 2000). Environmental conditions, such as temperature, oxygen level, absence of physiological barriers influence hatching (Perry, 1987; Perry, 1997). Cumulative percentage hatch of *M. incognita* was reported to be positively correlated with enzymes activity such as protinase and lipase (Perry *et al.*, 1992). However, enzymes activity does not appear to be related to plant signals (Perry, 1997). The observations of similar hatching in response to diffusates from mycorrhizal and non-mycorrhizal plants clearly exhibit that root diffusates did not influence nematodes hatch and thus the difference in galls incidence in the treatments does not mean that more juveniles hatched and attacked the root systems. Still, it is possible that hatching test in liquid suspensions does not permit a realistic assessment of all factors that may influence nematode hatch in soil. However, an *in vitro* test was adopted due to its efficacy and reduced error factor compared to *in situ* systems where re-extraction of the eggs and juveniles from soil are required.

### 3.2.3 J2 attraction to mycorrhizal and non-mycorrhizal plants

#### 3.2.3.1 Experimental set-up

The migration rate of the second stage juveniles of *M. incognita* toward mycorrhizal and non-mycorrhizal plants was evaluated in a pair choice assay (Olfactometer test). Each experimental unit consisted of two 2 l plastic pots attached to each other with a dark PVC tube for sewage discharge (Ostendorf, Germany). The tube was composed of four compartments (50 mm each) that were separated from each other by polyester gauze. The compartments were filled with fine washed and sterilized sand and then attached to the empty pots. Three days before inoculating with *M. incognita*, tomato plants grown with or without AMF (10% v/v) were transferred with their substrate to the experimental units in a way that always a mycorrhizal one was connected by the tube to a non-mycorrhizal one. Nematode inoculation (1200 J2 in 2ml of water) was done through a hole in the upper surface at the middle of the tube.

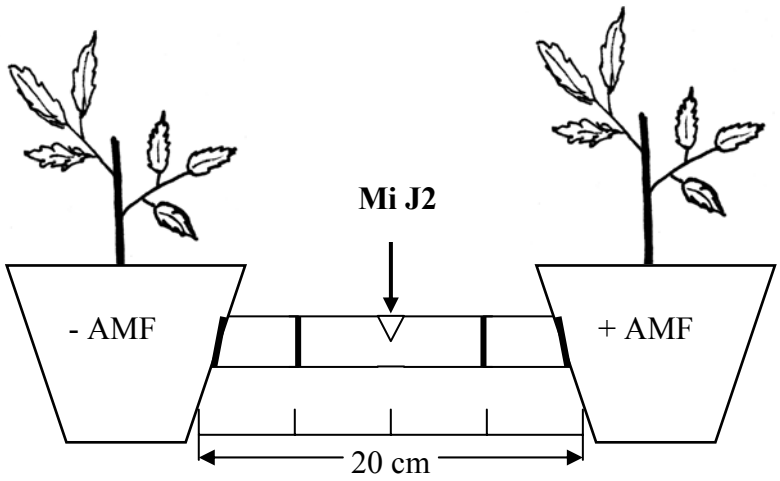


Figure 3.5: Diagram of the experimental unit in which attraction of J2 was studied.

In the first experiment, *G. intraradices* 510 was tested using five weeks old tomato plants (at flowering stage) and evaluation of juvenile migration took place 48 hours after inoculation. To confirm the result, the experiment was repeated using the AMF *G. etunicatum* 36, and *G. intraradices* 510, each separately, tomato seedlings used then were three weeks old, and evaluation of juvenile migration took place 24 hours after inoculation.

For the recovery of second stage juveniles, the sand in each compartment was mixed with 2 l of water and stirred for 30 seconds, then allowed to settle for 20 seconds. The upper suspension was passed through a set of 150, 50, 30 and 10 µm sieves respectively. The precipitated sand was subjected to this procedure five times. To recover the nematodes, the sieves were rinsed with 25-30 ml of water. Juveniles extracted from each compartment were counted separately in a nematode counting chamber under microscope (method modified; Sikora and Schuster, 2000). The recovery rate for each experimental unit was calculated as the number of juveniles recovered in relation to the number applied and expressed as percentage.

**3.2.3.2 Results**

In both experiments, including plants of different ages also at different harvest times, significantly higher numbers of *M. incognita* juveniles migrated towards non-mycorrhizal plants (Figure 3.6, a-c). There was no difference between *G. intraradices*

isolate 510 and *G. etunicatum* isolate 36 regarding this effect. This difference in migration was not attributed to differences in growth between the mycorrhizal and the non-mycorrhizal plants since no differences in this regard were observed (Table 3.5).

Table 3.5: Root fresh weight (R FWT), shoot fresh weight (Sh FWT), shoot dry weight (Sh DWT) and mycorrhizal colonization (MF, in % colonized root pieces of 1 cm length) ±SE of plants used in the attraction experiment, n = 6.

| **Treatments** | **R FWT [g]** | **Sh FWT [g]** | **Sh DWT [g]** | **MF [%]** |
|---|---|---|---|---|
| ***G. intraradices* 510 (5 wks)** | Not recorded | 93.7 ± 8.0 | 20.9 ± 0.7 | 70 ± 3.2 |
| **Non-mycorrhizal** | Not recorded | 98.3 ± 2.3 | 20.6 ± 0.46 | |
| ***G. intraradices* 510 (3 wks)** | 15.2 ± 1.6 | 51.7 ± 1.4 | 10.5 ± 1.2 | 62 ± 7.8 |
| **Non-mycorrhizal** | 16.5 ± 1.9 | 52.9 ± 1.4 | 11.0 ± 1.2 | |
| ***G. etunicatum* 36 (3 wks)** | 14.6 ± 3.0 | 44.1 ± 3.8 | 9.2 ± 1.3 | 55 ± 6.7 |
| **Non-mycorrhizal** | 15.6 ± 1.7 | 51.8 ± 3.1 | 10.6 ± 1.4 | |

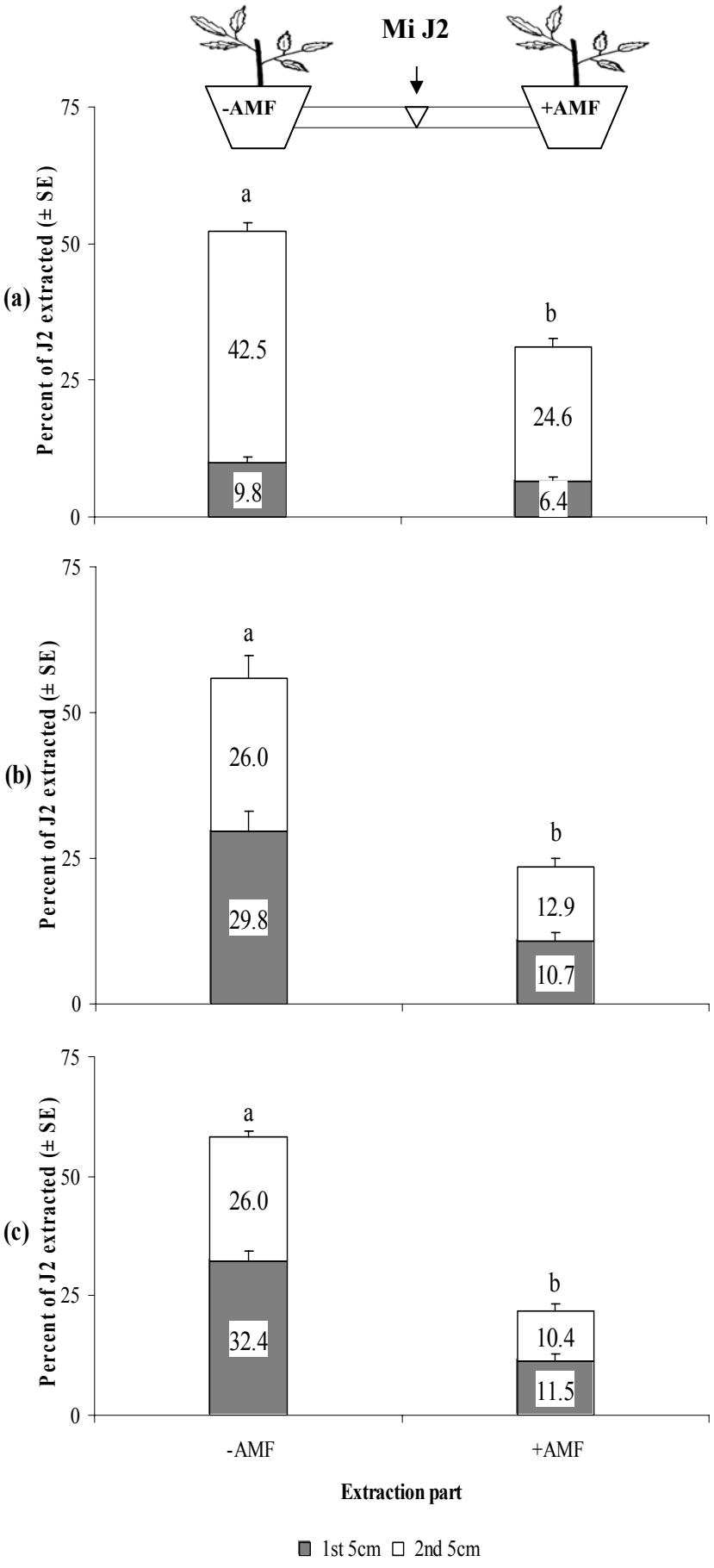


Figure 3.6: Orientation of *M. incognita* larvae in an olfactometer test: Percentage of nematodes extracted from each part is shown.

a) Attraction experiment I, AMF *G. intraradices* 510; plant age 5 wks; nematode extraction after 48 h.

b) Attraction experiment II, AMF *G. intraradices* 510; plant age 3 wks; nematode extraction after 24 h.

c) Attraction experiment II, AMF *G. etunicatum* 36; plant age 3 wks; nematode extraction after 24 h. Bars followed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

### 3.2.3.3 Discussion

The second event that might be altered in *M. incognita* - AM systems is the attraction of juveniles towards the roots. Generally, attractiveness of a host to a pest is correlated with the host or non-host status of the plant (Viglierchio, 1961; Perry, 1997). Attractiveness of roots depends on many parameters such as root age, level of growth activity, and presence of micro-organisms (Lownsbery and Viglierchio, 1961; Perry, 1997). In both sets of the attraction experiment presented here, non-mycorrhizal plants were more attractive than mycorrhizal ones. The difference in migration of *M. incognita* juveniles is attributed to an altered root growth of mycorrhizal plants as no difference in this regard was observed.

*Meloidogyne* second stage juveniles do not find their host by random movement, but are attracted to host in response to stimuli (Prot, 1980; Hussey, 1985). The primary mechanism of host finding behaviour by nematodes is believed to involve chemotactic factors emanating from the hosts' roots (Zuckerman and Jansson, 1984; Perry, 1997). AM fungal colonisation alters root exudates qualitatively and quantitatively, and changes in the exudation pattern are likely to alter the chemotaxis to the roots by the pathogens (Marschner, 1997; Harrier and Watson, 2004). Therefore, the decreased attractiveness (or the repellence) of the mycorrhizal roots could result from altered levels or quality of root exudates due to mycorrhizal colonization. It is reported that mycorrhizal infection decrease root exudation of sugars and amino acids and the formation of lipid-rich vesicles may increase costs of the mycorrhizal roots (Peng *et al.*, 1993; Marschner *et al.,* 1997).

Root exudates can be classified broadly according to their rates of diffusion in soil into three types; (i) volatile or gaseous compounds, (ii) water soluble and high diffusible components and (iii) non-diffusible materials (Spiegel *et al.*, 2003). Observations concerning the kairomones attracting nematodes point that they are of hydrophilic nature (Perry, 1997; Rühm *et al.*, 2003). The design of the experimental unit excludes the involvement of non-diffusible materials. Perry (1997) presumed that plant signals reach nematode sensory receptors (amphids) exclusively by diffusion. A genus specific glycoprotein -associated with the amphids found in several *Meloidogyne* species- was expressed in all stages of the *Meloidogyne* life cycles but not in the sedentary female which indicate the involvement of nematode sensory receptors in receiving plant signal and their role in host orientation (Davis *et al.*, 1992). It is suggested that nematodes ori-

entate along amino acids-, carbon dioxide-, lower redox potential- gradients around and at root's surface (Hussey, 1985; Perry, 1997). However, whether factors influencing long distance migration (>10 cm) are similar to factors influencing attraction to target tissue is unknown.

Lucas García *et al.* (2001) reported that exudates are related to plant age. The attractiveness of mycorrhizal plants did not change when mycorrhizal plants of different age were tested. Moreover, the two AMF isolates used in the experiments had an identical effect on nematode attraction, although *G. etunicatum* 36 could not reduce the numbers of nematode galls as observed earlier (in the results obtained from testing the influence of nursery AMF treatments on nematode infection and mycorrhizal colonization extension into AMF-free soil). This may reflect that even when AMF isolates do not have the same influence on nematode infection; they share the same basis of attractiveness and would exhibit that root finding behaviour by the nematodes does not necessarily reflects the degree of compatible interaction establishment.

Further analysis of this phenomenon requires fractionation and comparison of root exudates from mycorrhizal and non-mycorrhizal plants. This would also help to determine whether decreased attractiveness was due to altered levels of exudates or to the generation of novel chemicals associated with the mycorrhizal interaction and capable to impair the orientation of nematodes. Still, the results of the attraction test represent a pair choice situation and do not necessarily reflect the situation in the field where the nematode might not have the choice between mycorrhizal and non-mycorrhizal roots. It cannot explain the reduced number of galls after infection with AMF isolates *G. intraradices* 510 and *G. etunicatum* 139.

### 3.2.4 Quantification of juveniles' invasion

#### 3.2.4.1 Experimental set-up

The experiment compared invasion into mycorrhizal and non-mycorrhizal tomato roots by *M. incognita* juveniles. Newly germinated tomato plants were grown in 9.5 cm diameter vertically incubated petridishes with a hole in the upper edge, and pores for water percolation at the lower edge. The substrate of the mycorrhizal treatment was mixed with 10% (v/v) AMF inoculum while the substrate of the control plants was mixed with the same amount AMF-free expanded clay. The experimental units were later covered

with aluminium foil. Five days later, nematode treatments received 2 ml aliquots of *M. incognita* juveniles' inoculum (50 ± 2.5 $ml^{-1}$); control treatments received 2 ml water.

The experimental design included two treatments, with and without AMF (*G. intraradices* 510), and 5 harvest times. Five replicates from each treatment were harvested with three days intervals. At each harvest AMF colonization and nematodes penetration rate were determined (Figure 3.7).

For staining nematodes within the root tissues; roots were bleached in 10% KOH (w/v), heated at 70°C for one hour and left in the KOH solution overnight, rinsed with tap water, and acidified with 1% HCl for 5 min. The roots were then stained in a solution of 10% blue ink in 5% acetic acid.

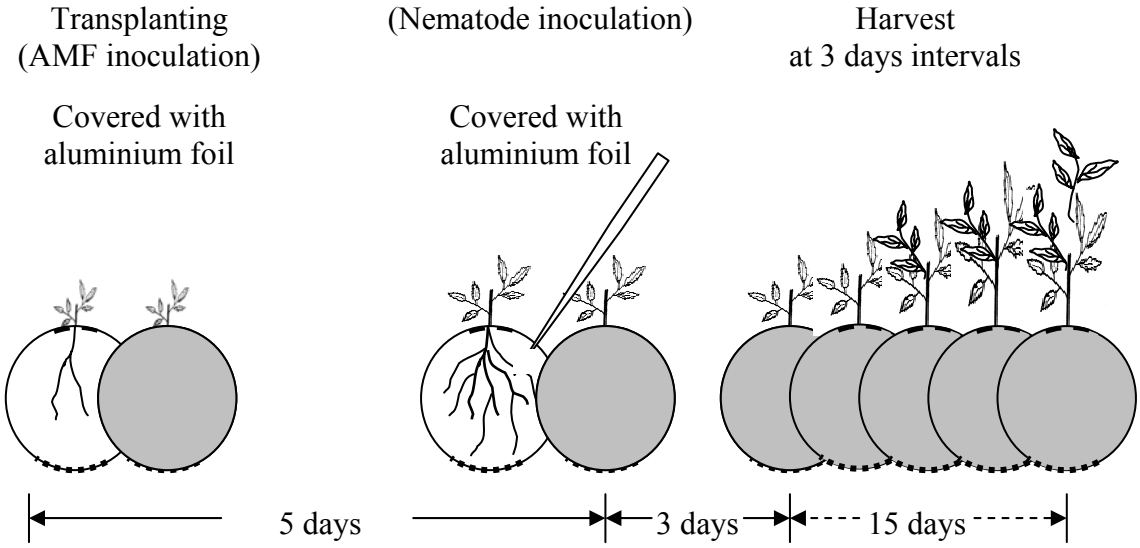


Figure 3.7: Diagram of the experimental unit in invasion of J2 was studied and an overview of the time frames used.

### 3.2.4.2 Results

Significantly fewer nematodes were detected in root of the mycorrhizal treatments at 3 and 6 days after inoculation. Later, 9 days and afterwards, no significant differences in invasion of mycorrhizal plants compared to non-mycorrhizal were detected. No differences in root weight were observed (Table 3.6).

Table 3.6: Invasion of *M. incognita* juveniles into mycorrhizal and non-mycorrhizal roots. Number of juveniles per plant detected in roots of *G. intraradices* 510 or non-inoculated Control, frequency of mycorrhizal colonization (MF, in % colonized root pieces of 1 cm length) and root fresh weight (R FWT) ±SE, n=5.

| Day | Penetration | | | | MF | R FWT [g] | |
|---|---|---|---|---|---|---|---|
| | Control | | *G. intraradices* 510 | | | Control | *G. intraradices* 510 |
| 3 | 11.2±1.7 | **a** | 5.8±0.6 | **b** | 28.0±3.7 | 1.40± 0.03 | 1.34± 0.02 |
| 6 | 19.2±2.4 | **a** | 10.8±1.1 | **b** | 26.0±5.1 | 1.39± 0.03 | 1.37± 0.04 |
| 9 | 26.4±4.3 | | 20.6±2.5 | | 42.0±5.8 | 1.46± 0.02 | 1.48± 0.04 |
| 12 | 27.4±2.3 | | 23.4±1.5 | | 46.0±5.1 | 1.49± 0.03 | 1.49± 0.06 |
| 15 | 28.6±1.6 | | 24.0±1.3 | | 44.0±7.5 | 1.52± 0.04 | 1.46± 0.05 |

Values in the same row followed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

### 3.2.4.3 Discussion

Nematode resistance due to pre-infection factors may occur at the root surface thereby influencing penetration (Thomson Cason *et al.* 1983); the assay was performed to determine whether the rate of juveniles invading mycorrhizal and non-mycorrhizal roots is in correspondence with the results obtained in the attraction test. The experimental unit used is expected to exclude long distance attraction.

Mycorrhizal colonization did not limit potential infection sites for nematode juveniles and the final number invading the roots of mycorrhizal plants was similar to those in non-mycorrhizal plants. These results corroborate with those of Smith *et al.* (1986). However, there was a clear delay in the invasion of mycorrhizal plants. Non-mycorrhizal roots were invaded faster. This suggests that juveniles reached or recognized penetration sites in non-mycorrhizal root earlier or that penetration was easier, whereas in mycorrhizal roots they required longer time to select a penetration site or to penetrate.

When second stage juveniles contact the plant root, they explore the root by rubbing and pressing the epidermal cells until locating an invasion site, likely in the elongation zone of the roots tips. The subventral glands are at their maximum size at this stage (Bird,

1968). During invasion, the juveniles secrete cell wall-degrading enzymes such as 1,4-ndoglucanases, pectates, and cellulases which are produced in the oesophageal glands (Rosso *et al.*, 1999; Huang *et al.*, 2003). Hence, entering the root involves a combination of mechanical piercing by the stylet and enzymatic softening.

The delayed penetration of mycorrhizal roots could thus be attributed to increased lignification of the cell walls of mycorrhizal roots (Gnavi *et al.*, 1996; Slezack *et al.*, 1999) and thus longer time required for penetration, since invasion involves mechanical and enzymatic actions (Hussey, 1985; Wyss, 1992; Francl, 1993, Huang *et al.*, 2003).

Delayed penetration may also be attributed to the interference of AM with recognition processes (Oka *et al.*, 2000). Considering the results obtained earlier (attraction assay), plants signals emanated from the mycorrhizal roots may have irritated the juveniles so that orientation of the penetration sites was delayed. If this to be proved, then factors influencing long distance migration (>10 cm) are similar to factors influencing attraction to target tissue.

Moreover, at such a high inoculation level, the extraradical mycorrhizal hyphae may have contributed - as physical barriers for juveniles' movements- in the delayed invasion. The size of the root system could be excluded from being involved here by showing that treatments had identical root weight.

The results obtained indicate that the less gall incidence in mycorrhizal plants, inoculated with *G. intraradices* 510 in earlier experiments, appears to be expressed primarily due to post infectional resistance mechanisms rather to differences in J2 penetration since no difference in the final penetration was observed. Moreover, if delayed penetration is proved to be a general phenomenon for all mycorrhizal plants, then the smaller gall size on mycorrhizal plants can be attributed to this aspect.

### 3.2.5 The histology of the AMF - nematode- tomato interaction

In order to follow up the development of nematodes after invasion and plant response after the infection process, preliminary histological investigations on tomato root tissue were carried out with detail studies of giant cells.

### 3.2.5.1 Experimental set-up

Histological differences in galls induced by *M. incognita* as affected by mycorrhizal inoculation were examined. Two tomato cultivars known to exhibit different susceptibility levels to RK, Hildares and Tiptop (Masadeh, 2000) and cultivar King kong II were used in this test. Two harvest times were considered

For each cultivar the were three treatments (two AMF isolates: *G. intraradices* 510, *G. etunicatum* 36, and a control); and two harvest times, 14 and 28 days after nematode inoculation (dai). At each harvest time, galls induced in tomato roots of different treatments were counted and classified into size scales (small, medium and large). Numbers of eggsacs were recorded only at the second harvest.

With regard to the preparation of galls for histological assessment, 15 galls were collected and were fixed for 48 hours in EFA (90:5:5 mixture of ethanol 70%, acid free formaldehyde solution 37%, and acetic acid). The fixed samples were dehydrated in a series of ethanol concentrations (70%, 90%, 96%, 100%). The embedding procedure in 2-hydroxyethyl-methacrylate (GMA) was done with Technovit 7100 (Hereaus Kulzer Comp., Wehrheim). The dehydrated gall samples were then soaked for two hours in a mixture of equal amounts of the basic Technovit 7100 solution and 100% ethanol. Overnight, the galls were placed in a mixture of 100 ml Technovit 7100 and 1g “Hardener I” for infiltration. For embedding and block formation, the infiltrated samples were orientated in wells of a special Teflon form (Histoform S). The base of the form was filled with embedding solution (15ml infiltration solution + 1ml “Hardener II”), then five galls were placed longitudinally, the rest of the embedding form was then carefully filled. After polymerisation at 37C° the sample blocks were removed from the form by use of the Technovit 3400 system and preserved at 4°C until sectioning.

Thin sectioning was done with a rotation microtome (Autocut 2040, Reichert-Jung Comp.). Two sectioning thickness were adopted; 4 µm and 6 µm for the two harvesting dates (14 and 28 days after nematode inoculation, respectively). The sections were then placed on water and every fourth continuous section was mounted on a glass slide, which was left overnight on a warm plate (48°C). The dried slides were stained with 0.1 % toluidine blue in 50 % ethanol for 12-15 min. Galls were compared at two harvest dates (14, 28 days after nematode inoculation) for numbers of induced giant cells and

numbers of nuclei per feeding site. Additionally the dimensions of the feeding site and females and average diameters of nuclei were measured.

### 3.2.5.2 Results

The tested tomato cultivars differed in their susceptibility to *M. incognita* as reflected by the number of galls induced (Table 3.7). The two cultivars Hildares and Kingkong II were more susceptible compared to cv. Tiptop. Inoculation with either AMF isolate, *G. intraradices* 510 or *G. etunicatum* 36 did not influence gall numbers in this experiment. Colonization among the mycorrhizal treatments did not differ (Table 3.8). No influence of the mycorrhizal treatments on gall size was observed (data not shown). Histological examination of the structure of galls showed that the observed difference in susceptibility was expressed as retardation in the development of the typical giant cell wall alterations (Figure 3.8). Cell wall alterations were clearly delayed in cv. Tiptop compared to cv. Hildares and cv. Kingkong II. Among other histological parameters quantified in this study (number of giant cells/feeding site, diameter of the nuclei, and the female size), no specific histological changes could be attributed to mycorrhization (Table 3.9).

Table 3.7: Number of galls and eggsacs per root system (±SE) induced by *M. incognita* at different harvest times (dai = 14, 28) on three tomato cultivars inoculated and non inoculated with *G. intraradices* 510 and *G. etunicatum* 36, n=3.

| **Cultivar** | **[dai]** | **Hildares** | | **Kingkong II** | | **Tiptop** | |
|---|---|---|---|---|---|---|---|
| **Treatment** | | **galls** | **eggsacs** | **galls** | **eggsacs** | **galls** | **eggsacs** |
| ***M. incognita*** | 14 | 296.3 ± 7.4 | | 295.7 ± 3.7 | | 145.0 ± 11.5 | |
| | 28 | 373.7 ±17.1 | 323.3 ± 23.4 | 327.0 ± 18.3 | 299.3 ± 9.2 | 219.3 ± 13.3 | 134.3 ± 6.9 |
| ***G. intraradices* 510** | 14 | 205.0 ± 35.6 | | 263.7 ± 2.8 | | 150.0 ± 11.2 | |
| | 28 | 344.0 ± 13.1 | 280.7 ± 12.2 | 326.0 ± 22.2 | 308.0 ± 21.7 | 243.7 ± 12.4 | 127.7 ± 16.3 |
| ***G. etunicatum* 36** | 14 | 312.3 ± 35.8 | | 297.0 ± 10.4 | | 129.0 ± 16.5 | |
| | 28 | 318.3 ± 11.7 | 245.3 ± 10.8 | 324.3 ± 11.3 | 307.7 ± 6.7 | 219.3 ±9.5 | 110.0 ± 15.8 |

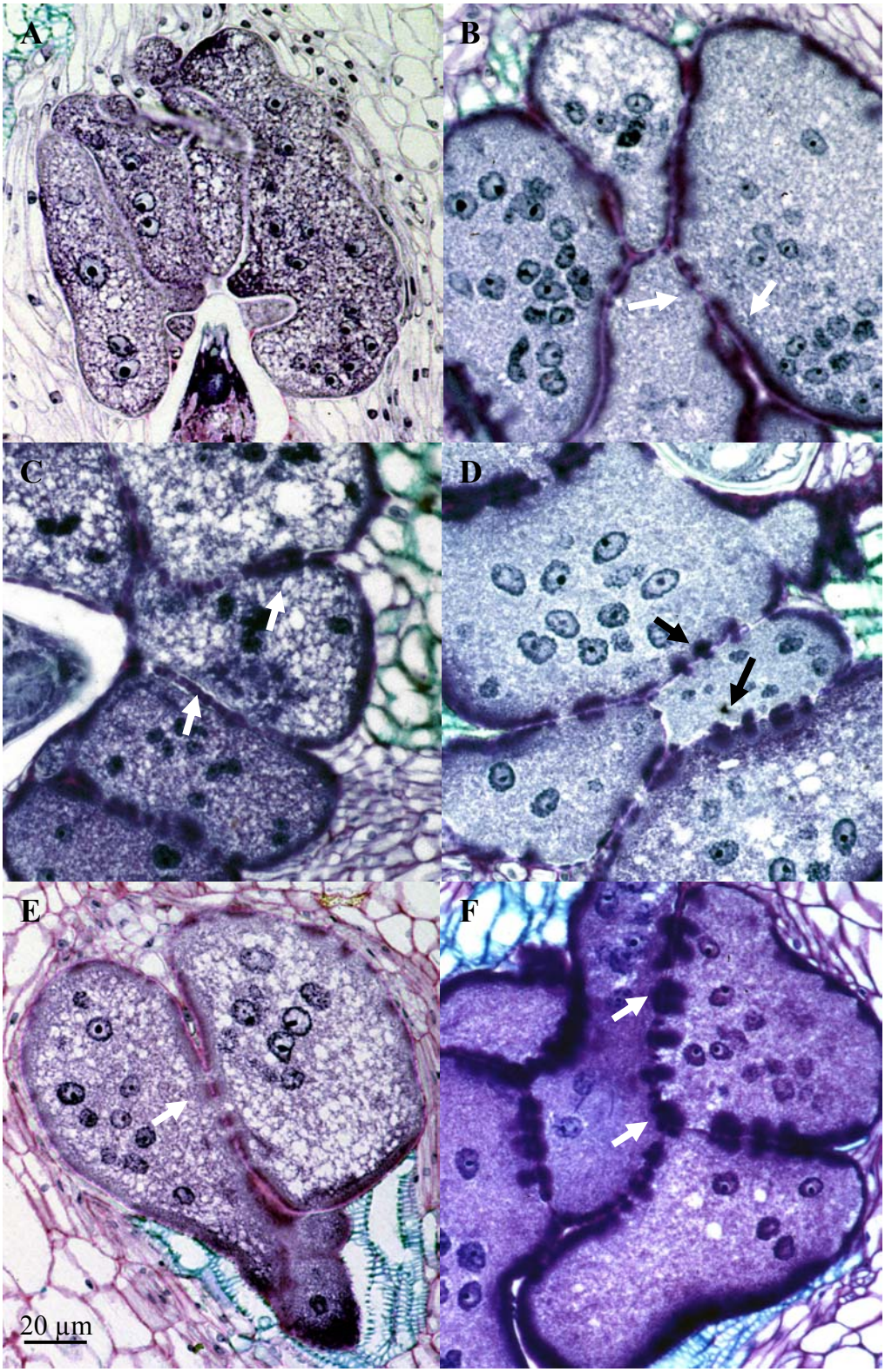


Figure 3.8: Giant cells induced by *M. incognita* in the tomato cultivars Tiptop (A and B), Hildares (C and D) and Kingkong II (E and F). 14 days (A, C and E) or 28 days (B, D and F) after inoculation with juveniles. Arrows = cell wall modifications.

Table 3.8: Mycorrhizal colonization frequency measured in the histological studies of the AMF-nematode-tomato-interaction. Hildares, King Kong II, Tiptop = tomato cultivars, dai= days after nematode inoculation, n=3.

| Cultivar | *G. intraradices* 510 + *M. incognita* | | *G. etunicatum* 36 + *M. incognita* | |
|---|---|---|---|---|
| | **14 dai** | **28 dai** | **14 dai** | **28 dai** |
| **Hildares** | 61.7 ± 8.8 | 88.3 ± 4.4 | 65.0 ± 5.7 | 78.3 ± 3.4 |
| **Kingkong II** | 68.3 ± 7.3 | 78.3 ± 6.0 | 55.0 ± 8.7 | 88.3 ± 3.4 |
| **Tiptop** | 73.3 ± 16.9 | 90.0 ± 2.9 | 81.7 ± 1.7 | 88.3 ± 1.7 |

Table 3.9: Parameters measured in the histological studies of the AMF-nematode-tomato-interaction (average of 15 galls). Hildares, King Kong II, Tiptop = tomato cultivars, dai= days after nematode inoculation, Area = length × width, n=15.

| | Treatment | dai | feeding site characteristics | | | female |
|---|---|---|---|---|---|---|
| | | | Giant cells per gall | diam. of nuclei [µm] | area [mm²] | area [mm²] |
| **Hildares** | ***M. incognita*** | **14** | 5.38 ±1.7 | 14.12 ± 3.9 | 0.13 ±0.04 | 0.05 ±0.02 |
| | | **28** | 5.62 ±2.3 | 14.43 ± 4.1 | 0.16 ±0.07 | 0.15 ±0.06 |
| | ***G. intraradices* 510 + *M. incognita*** | **14** | 6.33 ±2.5 | 13.92 ± 3.7 | 0.16 ±0.08 | 0.04 ±0.03 |
| | | **28** | 6.15 ±2.3 | 14.70 ± 4.8 | 0.21 ±0.07 | 0.18 ±0.06 |
| | ***G. etunicatum* 36 + *M. incognita*** | **14** | 5.70 ±2.1 | 11.61 ± 3.7 | 0.17 ±0.07 | 0.04 ±0.03 |
| | | **28** | 5.64 ±2.1 | 15.37 ± 5.1 | 0.20 ±0.09 | 0.17 ±0.06 |
| **King Kong II** | ***M. incognita*** | **14** | 5.75 ±1.2 | 13.29 ± 3.4 | 0.15 ±0.03 | 0.06 ±0.03 |
| | | **28** | 5.71 ±1.1 | 12.55 ± 4.1 | 0.14 ±0.05 | 0.16 ±0.03 |
| | ***G. intraradices* 510 + *M. incognita*** | **14** | 5.64 ±1.0 | 12.70 ± 3.7 | 0.14 ±0.05 | 0.05 ±0.03 |
| | | **28** | 5.87 ±0.7 | 13.84 ± 4.4 | 0.14 ±0.04 | 0.16 ±0.04 |
| | ***G. etunicatum* 36 + *M. incognita*** | **14** | 5.80 ±1.5 | 13.57 ± 3.6 | 0.15 ±0.05 | 0.05 ±0.02 |
| | | **28** | 5.73 ±1.0 | 12.19 ± 3.3 | 0.17 ±0.07 | 0.16 ±0.04 |
| **Tiptop** | ***M. incognita*** | **14** | 5.80 ±1.2 | 13.17 ± 4.4 | 0.15 ± 0.05 | 0.02 ±0.01 |
| | | **28** | 6.29 ±1.4 | 15.02 ± 6.8 | 0.14 ±0.06 | 0.10 ±0.06 |
| | ***G. intraradices* 510 + *M. incognita*** | **14** | 5.76 ±1.4 | 12.55 ± 3.5 | 0.11 ±0.03 | 0.02 ±0.01 |
| | | **28** | 5.67 ±1.0 | 13.21 ± 4.0 | 0.15 ± 0.05 | 0.13 ±0.05 |
| | ***G. etunicatum* 36 + *M. incognita*** | **14** | 6.13 ±1.3 | 13.45 ± 3.7 | 0.15 ±0.05 | 0.02 ±0.01 |
| | | **28** | 5.2 ±1.3 | 15.57 ± 4.2 | 0.17 ±0.07 | 0.15 ±0.07 |

### 3.2.5.3 Discussion

The cellular response following root-knot nematode invasion of different hosts has been studied (Bleve-Zacheo *et al.*, 1998; Rodrigues *et al.*, 2000). The morphology of the cells acting as the feeding site or located near the feeding site is different from other cells; differences include the nucleus, the cytoplasm, and the cell wall. lobing of nuclei, proliferation of cytoplasm and organelles, and development of irregularly thickened cell walls, are indicators of increased metabolic activity.

In spite of AMF absence on nematode infection parameters, the histological examination was undertaken to compare the cellular reactions occurring on roots of the different cultivars. The histological investigations of galls showed variability in the response of the cultivars regarding RKN infection. Although all were susceptible hosts, reactions varied with plant cultivars but not with AMF isolates used. When the nematode invades the root tissue and becomes sedentary, the initial giant cells are transformed into a multinuclear system of transfer cells of high metabolic activity (Bleve-Zacheo and Melillo, 1997). Initial plant responses to nematode infection include cell wall thickening and deposition of lignin (Endo, 1991). Between the cells, the walls are transformed by material deposited between high numbers of plasmodesmata (Huang and Maggenti, 1969). Nutrients delivered to the nematode by the giant cell system are transported via the phloem and loaded into the giant cells (Grundler and Böckenhoff, 1997) probably by sucrose carriers (Juergensen *et al.*, 2003), a process crucial for the functioning of the giant cell system, and referred to as “sink phenomenon” (Hussey, 1985). The assumed sink competition by the neighbouring mycorrhizal fungus had no influence on the characteristics of the nematodes feeding site in this study, i.e. cell number or size. However, there are reports concerning influence of mycorrhizae on the characteristics of feeding sites induced by RKN. Sikora (1979) reported that giant cells produced on mycorrhizal tomato plants inoculated with *Glomus mosseae* were smaller, fewer in number and contained less nuclei and denser cytoplasm than giant cells of the same age produced on non-mycorrhizal tomato plants.

The tomato cultivars Hildares and Tiptop are known to have different susceptibility levels to *M. hapla* (Masadeh, 2000). The results presented here suggest that differences in feeding site characteristics are related to different degrees of susceptibility. Studying the histological and ultrastructral changes in coffee in response to *Meloidogyne exigua* and

*M. megadora*, Rodrigues *et al.* (2000) indicated that differences in morphological characteristics of nematode feeding sites can be in relation to resistance and susceptibility. Coffee root cells fed upon by *M. megadora* showed retraction and roughening of the inner wall surface, great presence of paramural bodies, and processes of autophagy within the vacuoles, all suggesting an intermediate response between susceptibility and resistance.

### 3.2.6 Mechanisms of action:

#### 3.2.6.1 Induced resistance

In the experiments studying the influence of nursery AMF treatments on nematode infection and mycorrhizal colonization extension into AMF-free soil the treatments of *G. intraradices* 510 yielded less gall numbers. Certain AMF isolates are capable to induce a systemic response in plants that would be antagonistic to the development of nematodes. This study was undertaken to test ability of *G. intraradices* isolate 510 to stimulate induced resistance against *M. incognita* was tested in a split-root trail.

##### 3.2.6.1.1 Experimental set-up

Two-weeks-old tomato seedlings were uprooted; roots were washed free of soil and carefully split into two halves with a dissecting scalpel until just below the cotyledons. Each half was transplanted into separate 400 ml plastic pot. The pots were attached together on the outside with tape. The AM fungus was inoculated to one side of split root systems; care was taken to keep the halves separated during transplanting. For the non-mycorrhizal treatment, one side of split root systems received the same amount of AMF- free expanded clay. One week after seedling establishment the untreated halves of the root system were inoculated with 500 freshly hatched juveniles.

##### 3.2.6.1.2 Results

In the split root experiment, inoculation of *G. intraradices* 510 did not influence growth parameters (table 3.10). However, the non-mycorrhizal root halves of mycorrhizal plants showed a significant reduction in numbers of galls, but not in number of eggsacs neither in gall diameter (table 3.11).

Table 3.10: Shoot fresh weight (Sh FWT), shoot dry weight (Sh DWT), root fresh weight (R FWT), and root length (±SE) of plants in a split root experiment. Gi 510 = *G. intraradices* 510, Mi = *M. incognita*, n=6.

| | | | | R FWT | | R L | |
|---|---|---|---|---|---|---|---|
| **Left pot** | **Right pot** | **Sh FWT** | **Sh DWT** | **left pot** | **right pot** | **left pot** | **right pot** |
| Control | Mi | 111.1 ± 3.3 | 21.1 ± 0.4 | 12.2 ± 0.7 | 16.1 ± 1.1 | 36.5 ± 2.1 | 38.9 ± 1.3 |
| *G. intraradices* 510 | Mi | 107.3 ± 2.3 | 22.1 ± 0.5 | 11.9 ± 1.0 | 13.3 ± 1.1 | 37.9 ± 1.6 | 38.3 ± 2.1 |

Table 3.11: Numbers of galls, numbers of eggsacs per root compartment, diameter of galls (±SE), and mycorrhizal colonization (±SE) in a split root experiment. Gi 510 = *G. intraradices* 510, Mi = *M. incognita*, MF = mycorhizal frequency in % colonized root pieces of 1 cm length, n=6.

| **Left pot** | **Right pot** | **No. of galls** | **No. of eggsacs** | **Gall diameter** | **MF %** |
|---|---|---|---|---|---|
| Control | Mi | 324.6 **a** ± 7.9 | 192.0 ± 20.9 | 1.01 ± 0.01 | |
| *G. intraradices* 510 | Mi | 279.3 **b** ± 9.7 | 205.0 ± 25.6 | 1.03 ± 0.04 | 84.0 ± 3.27 |

Values in the same column followed by different letters are significantly different according to Tukey's multiple range test ($P \leq 0.05$).

#### 3.2.6.1.3 Discussion

The split root experiment presented here was used to elucidate the possible involved mechanism of nematode suppression by mycorrhization. In this context, gall number after inoculation with juveniles was reduced only in the non-mycorrhizal halves of AMF treatment. However, the level of reduction did not reach the suppression found in the mesh pot experiment, where nematodes had been inoculated as eggs giving a lower invasion pressure over a longer period. Which suggest that nematode suppression due to mycorrhizal inoculation is a result of both localized defense response in the colonized

tissue and systemic responses in the non-mycorrhizal roots. It is likely that AM are acting by more than one mechanism as biocontrol agents. Regarding soil-borne diseases both local effects and induced resistance have been reported from mycorrhizal tomato plants colonized by *G. mosseae* BG 12 (Cordier *et al.*, 1998).

Plant responses to induced resistance by AMF may include changes in root physiology, carbon allocation, root exudation and morphology. The activation of specific plant defence mechanisms as a response to AM colonization has been reported and reviewed. Root infection by VAM can elicit the production phytoalxins and associated isoflavinoid; molecules that are usually associated with the development of host resistance to pathogens, these molecules diffuse from cell to cell and this ability may contribute to the irregular distribution of infected cells within cortical tissues. (Gianinazi, 1991)
However, this aspect of the interaction seems also to be very specific and highly dependent on the particular association of the plant cultivar, fungal species and isolate and nematode species since the results do not corroborate with earlier ones under similar conditions (Masadeh *et al.*, 2004).

## 4 Physiological markers for the interaction between *M. incognita* and AMF

**Abstract**

A greenhouse pot experiment was conducted to observe differences in susceptibility to nematode infection in roots non-colonized or colonized to different degrees by AMF. Pre-inoculation of tomato seedlings cv. King Kong II with the AM fungus *G. intraradices* 510 suppressed nematode infection. The degree of nematode infection suppression was not influenced by the initial inoculation level of AMF. Mycorrhizal treatments inoculated with either 1% or 5% v/v AM inoculum yielded similar number of nematode galls. The diameter of nematode galls and mycorrhizal colonization frequency were significantly influenced by AMF initial inoculation level. Neither mycorrhizal parameter was influenced by nematode inoculation.

Nematode inoculation caused, in correlation with the amount of nematodes used, an increase in proline content of roots. In leaves, raising the nematode inoculation level caused a decrease in the chlorophyll fluorescence parameter, Performance Index ($PI_{abs}$), the latter representing a physiological marker for plant vitality. Mycorrhization did not change the proline content, but had at least at early stages of the experiment a positive impact on photosynthetic activity, assessed as $PI_{abs}$.

### 4.1 Introduction

AMF and RKN are involved in major physiological processes in the plant, including host respiration, photosynthesis, nutrient translocation and availability, water relations. Thus, evaluating the interaction between the fungal endophyte and the sedentary nematode could be measured in other terms than the classical parameters of AMF influence on host efficiency and sensitivity. However, investigations of the biochemical and physiological relationships of AMF and RKN to their hosts have been rarely conducted.

Photosynthesis is of major importance in the life of plants; any interference, disturbance or stimulation of this process would influence plant growth and performance. It involves two linked stages: 1. the light reaction that occurs in the grana and starts with splitting the water at the photosystem II reaction centre and produces ATP and forms NADPH by transferring the electron from water to $NADP^+$, 2. the dark reaction that takes place

in the stroma and uses ATP and the electrons donated by the NADPH to form sugar from $CO_2$ .

Nematode infection causes plant chlorosis and stunted growth, mainly due to their influence on water and nutrients uptake, thus it is expected that photosynthesis is reduced in nematode infected plants (Melakeberhan and Webster, 1993). A decrease in the photosynthetic rate of *M. incognita* infected beans has been observed as early as three days after inoculation (Melakeberhan *et al.*, 1986). On the other hand, AMF is known to improve plant resistance or tolerance to nematodes, one of the proposed mechanisms is the improved water and nutrient uptake by AMF plants and improved rates of photosynthesis in AMF plants. Stimulated photosynthetic activities in mycorrhizal plants were reported and attributed mainly to improved phosphate status and increased synthesis of the photosynthetic pigments, which in turn result in higher rates of photosynthesis (Brown and Bethlenfalvay, 1987; Guillemin *et al*., 1996).

Another considerable impact of nematodes on plant physiology is their influence on contents of amino acids (Lewis and Mc Clure, 1975; Meon *et al.*, 1978; Showler *et al.*, 1991; Hassan *et al.*, 1994). Amino acid content may change with stress and appear to be linked with the susceptibility and resistance to some pests including nematodes (Lewis and Mc Clure, 1975; Stephan *et al.*, 1980; Showler *et al.*, 1991). Proline is a non-essential amino acid and the most rigid of the twenty naturally occurring amino acids. In some plants it appears to be critically important in the formation of cell wall proteins, and the extensibility of the cell wall may be controlled by the amount of hydroxylation of the proline residues in it (Lewis and Mc Clure, 1975). Galls induced on tomato roots in response to infection by root knot nematodes contain large amounts of free amino acids, particularly proline (Meon *et al.*, 1978).

The aim of this study was to test whether the influence of each, AMF and RKN separately and in combination, on the host plant can be qualified by investigating the photosynthetic activity through probing the behaviour of the photosystem II by means of chlorophyll-a-fluorescence emitted by host leaves did access to the photosynthetic activity (Strasser *et al.*, 2000). Another aim of the study was to test the influence of AMF on the concentration of proline as it is reported to be a stress marker and exists in large amounts in nematode infected plants and AM is known to influence its level.

## 4.2 Material and methods

### 4.2.1 Analysis of proline content

Roots were cut into 2 cm segments and 1 gm of fresh root tissue from each plant was washed with distilled water, wrapped with aluminium foil and immediately frozen in liquid nitrogen to be stored at -28°C until assay. For analysing the proline content in shoots, 5 to 7 leaflets were taken from the eighth leaf of each plant, wrapped in aluminium foil, immediately frozen in liquid nitrogen and stored at -28°C until assay.

Frozen roots and leaves were separately ground to a fine powder in liquid nitrogen with a porcelain mortar and pestle. Approximately 0.4 gm were used for proline extraction, 1.8 ml 10% sulfosalicylic acid was added. The mixture was then vortexed, and allowed to stand on ice for 30 min, then centrifuged at 10000 *g* for 15 minutes. 500 µl of the upper aqueous layer was removed into a boiling tube. 300-µl acetic acid, and 300 µl fresh ninhydrin reagent (25 mg/ml ninhydrin in acetic acid/phosphor acid mixture, end concentration: 60% acetic acid, orthophosphate acid 2.4 M) were applied. The samples were heated in a water bath for 45 min and then cooled to room temperature in ice water. The ninhydrate colour complex was then extracted with 2 ml toluene. The absorbance was measured photometrically at 520nm using toluene for a blank (Bates *et al.*, 1973). The proline concentration was estimated through a standard curve of L-proline with end concentrations of 0, 5, 10, 20, 40, and 60 µg (Figure 5.1).

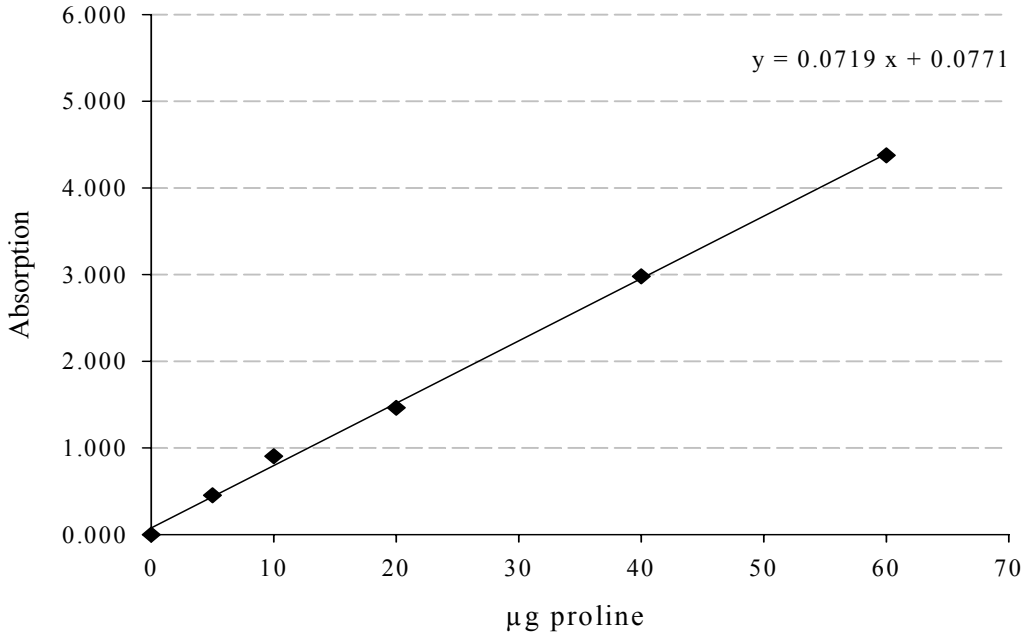


Figure 4.1: Standard curve of absorption at 520nm of L-proline solution in toluene.

### 4.2.2 Chlorophyll-a-fluorescence measurements

Plants were dark adapted for 45 min before measurements. Chlorophyll-*a*-fluorescence kinetics was measured using the Plant Efficiency Analyzer (Handy PEA, Hansatech Ltd., King's Lynn, Norfolk, UK); data were analysed and normalised with the program 'Biolyzer' (developed by R. Maldonado-Rodriguez, Laboratory of Bioenergetics of the University of Geneva). Measurements were conducted with six coeval plants on leaflets of mid leaf-levels. Chlorophyll-*a*-fluorescence was measured at various steps in the time course related to nematode inoculation and development (one week before inoculation, one week after nematode inoculation, then every second week until harvest); using an excitation light intensity of 2500 µmol $m^{-2}$ $sec^{-1}$ plant tissue was exposed to excitation light during 1 sec.

The behaviour of the photosystem was evaluated with the JIP-Test (Strasser *et al.*, 1999, 2000), of which two selected parameters were used in this study: First, the Performance Index ($PI_{abs}$) was chosen as a parameter accounting for functionality of both photosystems (PS II and I); it provides a general quantitative value of the actual state of plant vitality by combining several physiological events in favour of photosynthetic performance (The effects on $PI_{abs}$ result from the regulation of its components which are: TR/ABS = density of fully active reaction centres per chlorophyll; ET/TR= efficiency with which a trapped exciton moves an electron into the electron transport chain further than $Q_A$, and ET/ABS= the probability that an absorbed photon will move an electron into the electron transport chain). Additionally, the dissipation per excited reaction centre (DI/RC) was analysed as it represents the energy that is lost for feeding into the electron transport chain.

## 4.3 Experimental setup

Tomato seedlings cv. King Kong II were transplanted into 800 ml plastic pots. Two weeks after transplanting, when the seedlings had developed enough roots, they were inoculated with 0, 500, 1000 and 5000 freshly hatched (≤ 3-day-old) *M. incognita* juveniles. At the inoculation level of 1000 J2 the influence of mycorrhizal colonization level was tested by pre-inoculating tomato seedlings at transplanting with 1% or 5% v/v AMF inoculum (*G. intraradices* 510).

Treatments of the experiment were as follows:

1. non-inoculated control (C),
2. inoculated with 500 J2 *M. incognita* (Mi L1),
3. inoculated with 1000 J2 *M. incognita* (Mi L2),
4. inoculated with 5000 J2 *M. incognita* (Mi L3),
5. inoculated with 1 % v/v *G. intraradices* 510 (1% AMF),
6. inoculated with 1 % v/v *G. intraradices* 510 + 1000 J2 *M. incognita* (1% AMF Mi L2),
7. inoculated with 5 % v/v *G. intraradices* 510 (5%AMF),
8. inoculated with 5 % v/v *G. intraradices* 510 + 1000 J2 *M. incognita* (5% AMF Mi L2).

The treatments were laid out in a completely randomised design and each was replicated 6 times. Plants were harvested five weeks after nematode inoculation.

**4.4 Results**

Data obtained show that all plant growth characters were not affected by the initial inoculation levels of AMF. In contrast, initial nematode inoculation levels influenced root but not shoot growth. A significant increase in root weight was observed at the highest nematode inoculation level (Figure 4.2).

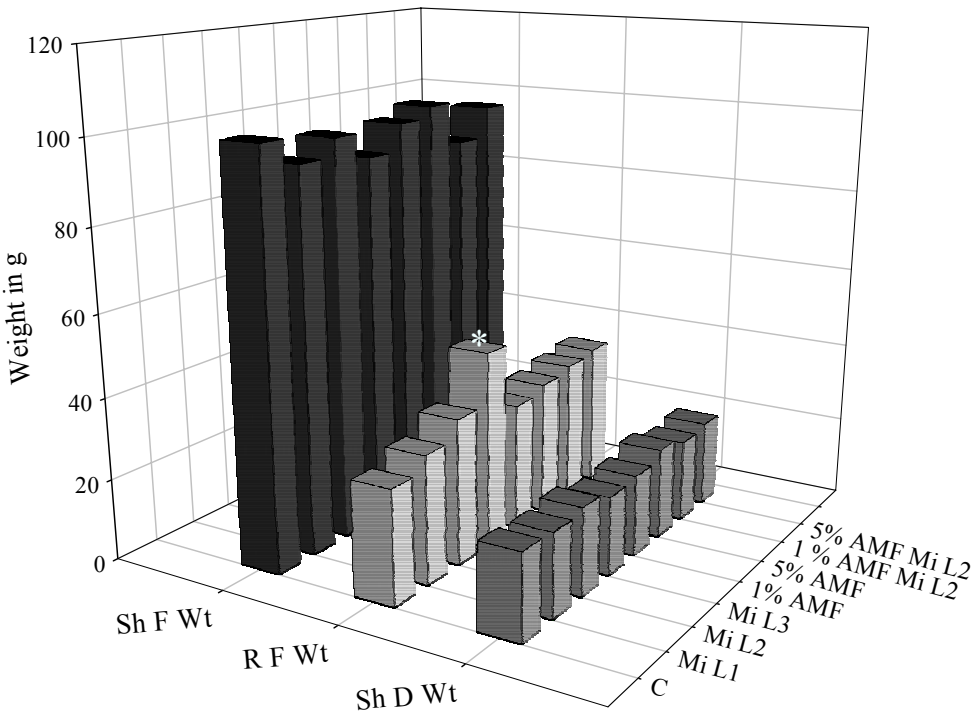


Figure 4.2: Shoot fresh weight (Sh F WT), root fresh weight (R F WT), and shoot dry weight (Sh D Wt) of tomato plants. Treatments were: Control (C), inoculated with 500 J2 *M. incognita* (Mi L1), inoculated with 1000 J2 *M. incognita* (Mi L2), inoculated with 5000 J2 *M. incognita* (Mi L3), inoculated with 1 % AMF *G. intraradices* 510 (1% AMF), inoculated with 1 % AMF + 1000 J2 *M. incognita* (1% AMF Mi L2), inoculated with 5 % AMF *G. intraradices* 510 (5%AMF), inoculated with 5 % AMF + 1000 J2 *M. incognita* (5% AMF Mi L2), * = significantly different according to Duncan's multiple range test ($P \leq 0.05$), n = 6.

Nematode parameters were influenced by the initial inoculation levels. Gall indexes (Figure 4.3, a), numbers of nematode galls (Table 4.1), numbers of eggsacs (Table 4.2) and galls diameter (4.4, a), all exhibited an increase with increasing the nematode initial inoculation levels. Pre-inoculation of seedlings with the AM fungus *G. intraradices* 510

suppressed nematode infection. This suppression was significant when comparing the gall index (Figure 4.3 b) and the number of galls induced per root system (Table 4.1), but not the number of egg sacs (Table 4.2). The initial inoculation level of AMF did not influence the degree of nematode suppression when comparing number of nematode galls (Table 4.1) or gall index (Figure 4.3, b). Both mycorrhizal treatments yielded similar number of nematode galls. In contrast, the diameter of nematode galls was significantly influenced by the AMF initial inoculation density; galls induced on root system of the 5% AMF Mi L2 treatment (inoculated with 5 % v/v *G. intraradices* 510 + 1000 J2 *M. incognita*) were smaller than those induced on root systems of the 1% AMF Mi L2 treatment (inoculated with 1 % v/v *G. intraradices* 510 + 1000 J2 *M. incognita*) and both were smaller than galls of the nematode treatment Mi L2 (Figure 4.4, b). AMF colonisation frequency, but not intensity, was also dependent on the initial inoculation density (Figure 4.5, a and b). Neither mycorrhizal parameter was influenced by nematode inoculation.

Table 4.1: Number of galls per root system (± SE) induced by different levels of *M. incognita* on non-mycorrhizal tomato plants and by 1000 J2 on mycorrhizal plants pre-inoculated with different levels of the AM fungus *G. intraradices* 510.

| | ***M. incognita* inoculation level** | | | |
|---|---|---|---|---|
| **AMF inoculation level** | **500** | **1000** | | **5000** |
| **Non-mycorrhizal** | 355.0 ± 51.9 | 634.7 ± 45.3 | **a** | 2653.6 ± 196.7 |
| **1% *G. intraradices* 510** | | 435.3 ± 36.2 | **b** | |
| **5% *G. intraradices* 510** | | 437.1 ± 57.7 | **b** | |

Values in the same column followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$), n = 6.

Table 4.2: Number of eggsacs per root system (± SE) induced by different levels of *M. incognita* on non-mycorrhizal tomato plants and by 1000 J2 on mycorrhizal plants pre-inoculated with different levels of AM fungus *G. intraradices* 510, n = 6.

| | **Nematode inoculation level** | | |
|---|---|---|---|
| **AMF inoculation level** | **500** | **1000** | **5000** |
| **Non-mycorrhizal** | 137.3 ± 19.5 | 228.5 ± 23.7 | 719.9 ± 55.5 |
| **1%** | | 182.2 ± 15.7 | |
| **5%** | | 188.0 ± 29.0 | |

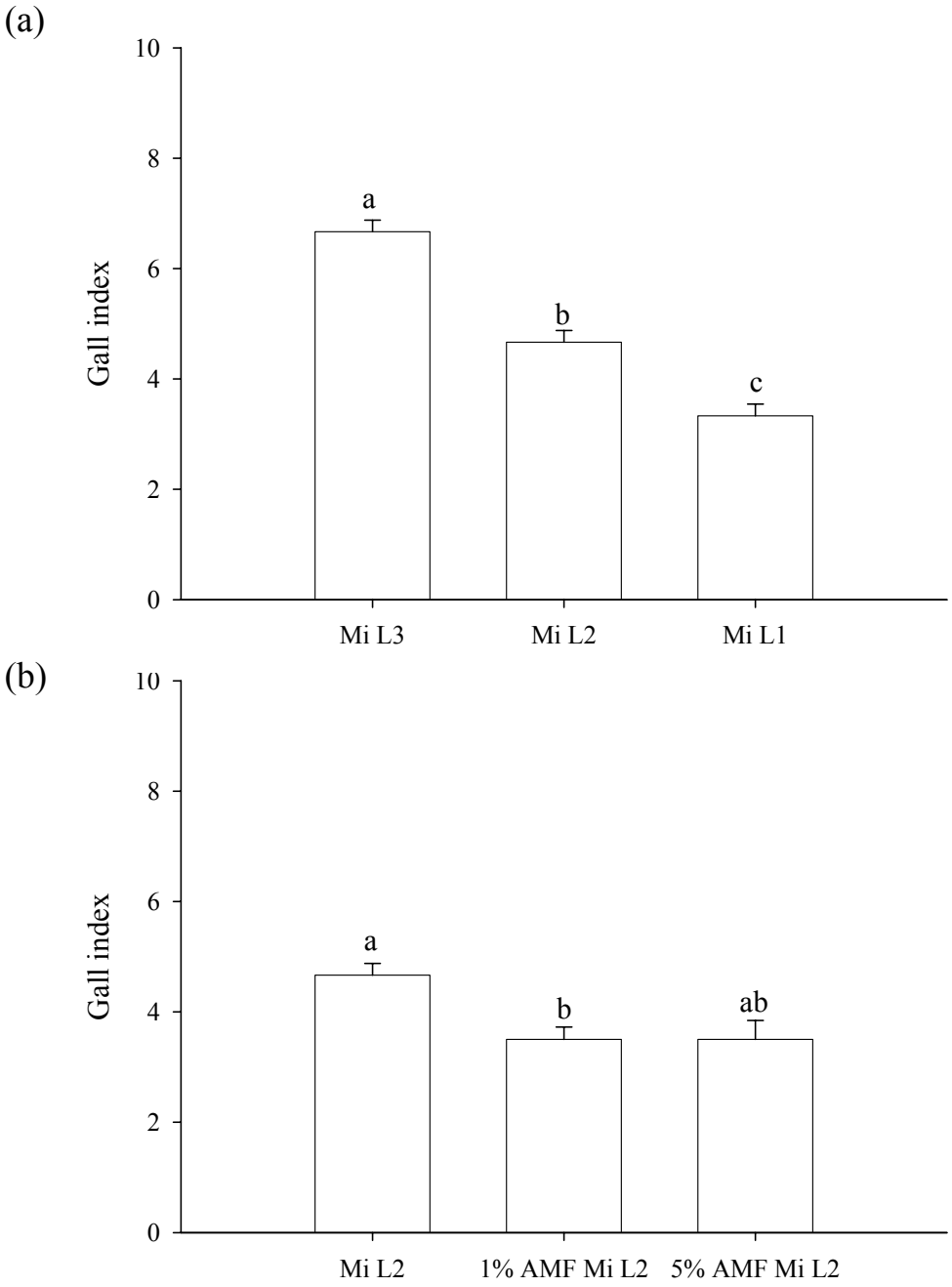


Figure 4.3: Galls indexes (±SE), (a) comparing three initial inoculation levels of *M. incognita*; 500 J2= Mi L1, 1000 J2= Mi L2, and 5000 J2 = Mi L3, (b) comparing the influence of three initial AMF (*G. intraradices* 510) inoculation levels and one nematode inoculation level; non-mycorrhizal= Mi L2; 1% AMF = 1% AMF Mi L2; 5% AMF= 5% AMF Mi L2. Bars headed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$), n=6.

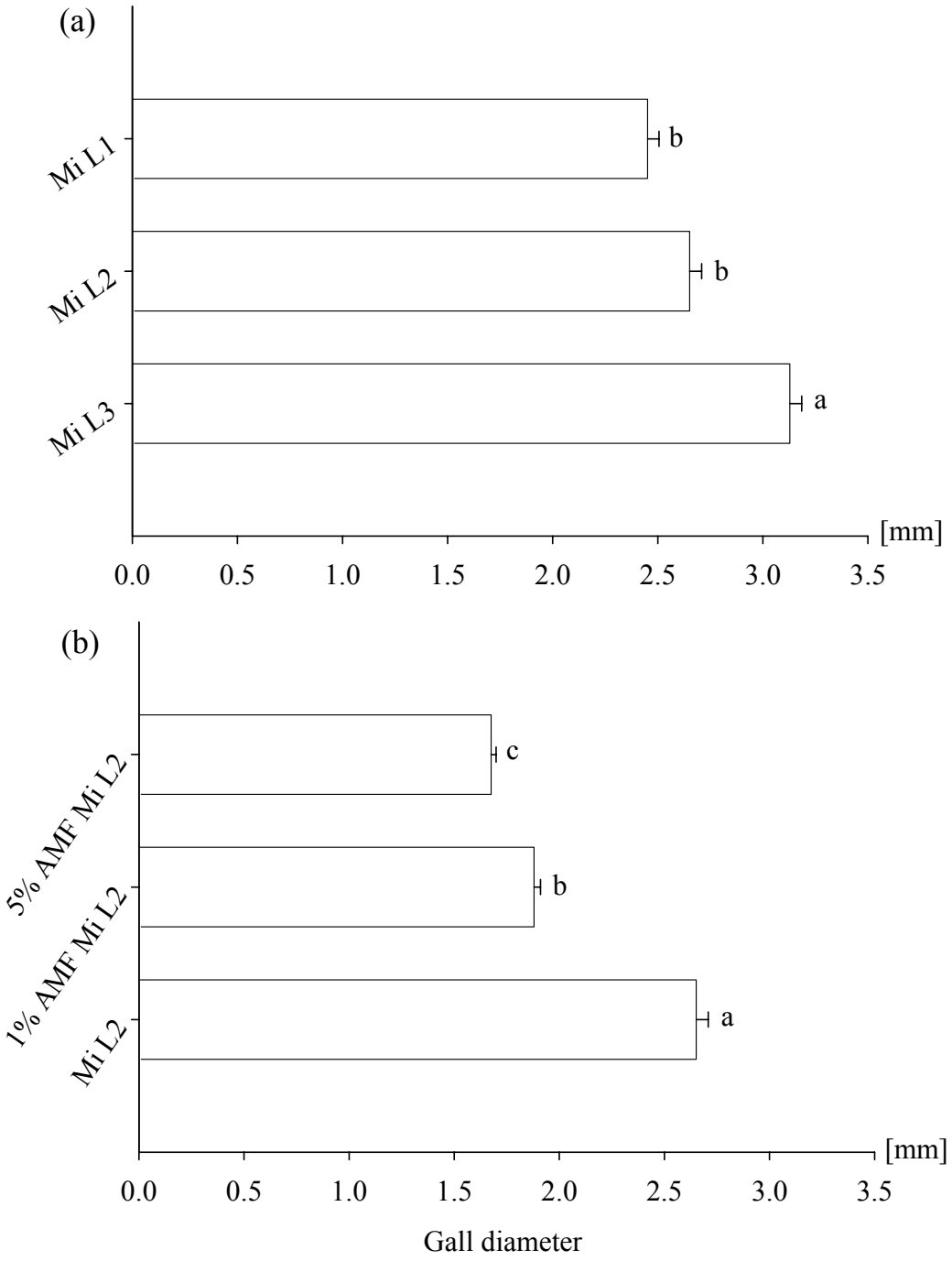


Figure 4.4: Diameter of galls (±SE), (a) compared at three levels of *M. incognita*; 500 J2= Mi L1, 1000 J2= Mi L2, and 5000 J2 = Mi L3, (b) compared at three initial AMF (*G. intraradices* 510) inoculation levels and one nematode inoculation level; non-mycorrhizal= Mi L2; 1% AMF = 1% AMF Mi L2; 5% AMF= 5% AMF Mi L2. Bars followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$), n=6.

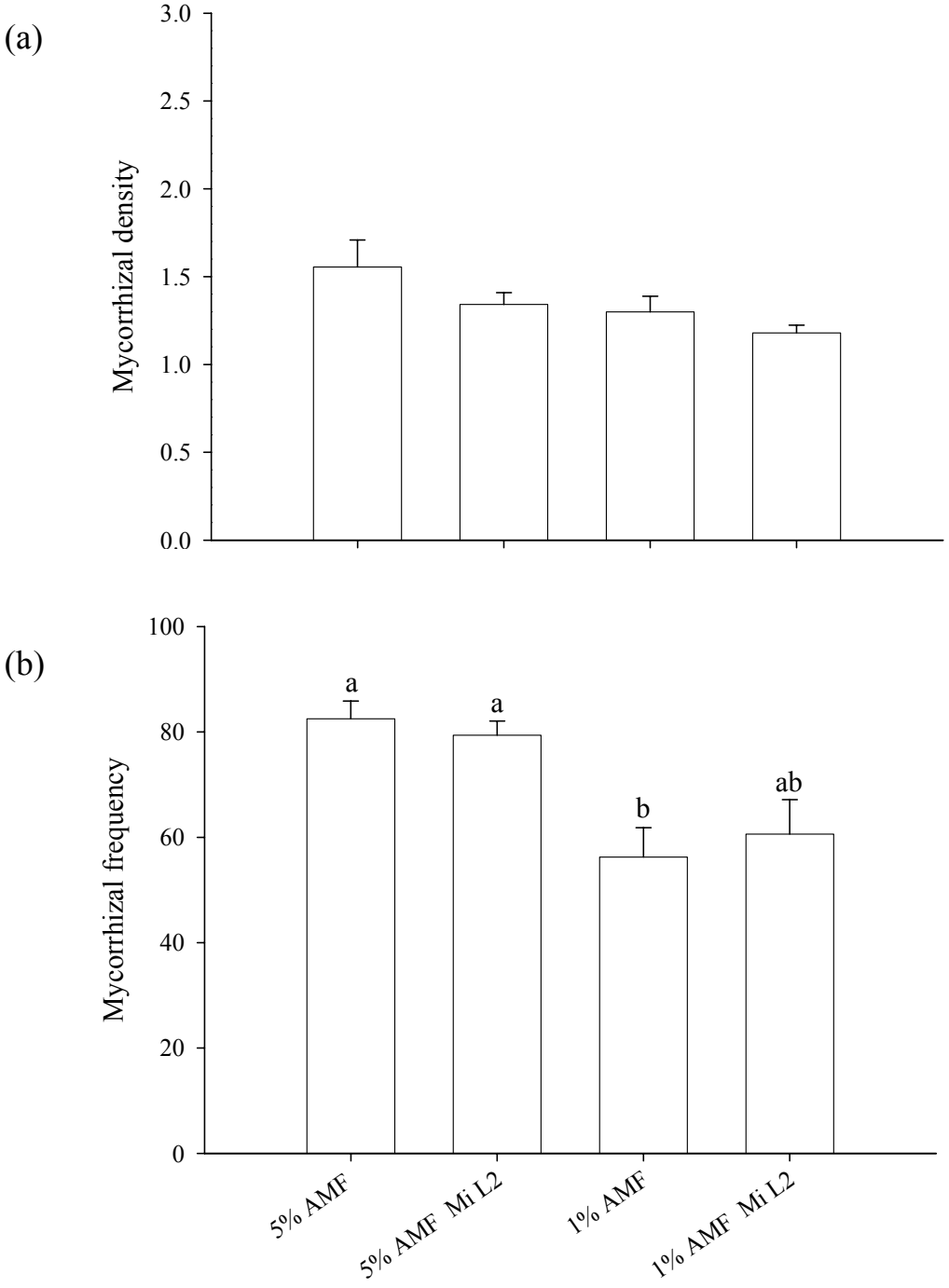


Figure 4.5: (a) Mycorrhizal density and (b) mycorrhizal frequency (±SE), as influenced by initial AMF inoculation level and *M. incognita*. Treatments are: 5 % *G. intraradices* 510 (5% AMF), 5 % *G. intraradices* 510 + 1000 J2 *M. incognita* (5% AMF Mi L2), 1 % *G. intraradices* 510 (1% AMF), 1 % *G. intraradices* 510 + 1000 J2 *M. incognita* (1% AMF Mi L2). Bars headed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$), n = 6.

Although values obtained from proline analyses were not significant, the data reveal a trend of higher proline content in roots compared to leaves (Figures 4.6, 4.7 and 4.8). Nematode inoculation caused an increase in proline content in tomato roots, the concentration increased with increasing density of nematode inoculum (Figure 4.6). Inoculation of healthy tomato plants with either level of the AM fungus *G. intraradices* 510 did not influence proline concentration neither in leaves nor in roots (Figure 4.7). Pre-inoculation of nematode infected plants with AMF seemed to reduce the concentration of proline in roots compared to the nematode treatments, however, with statistically insignificant differences (Figure 5.8).

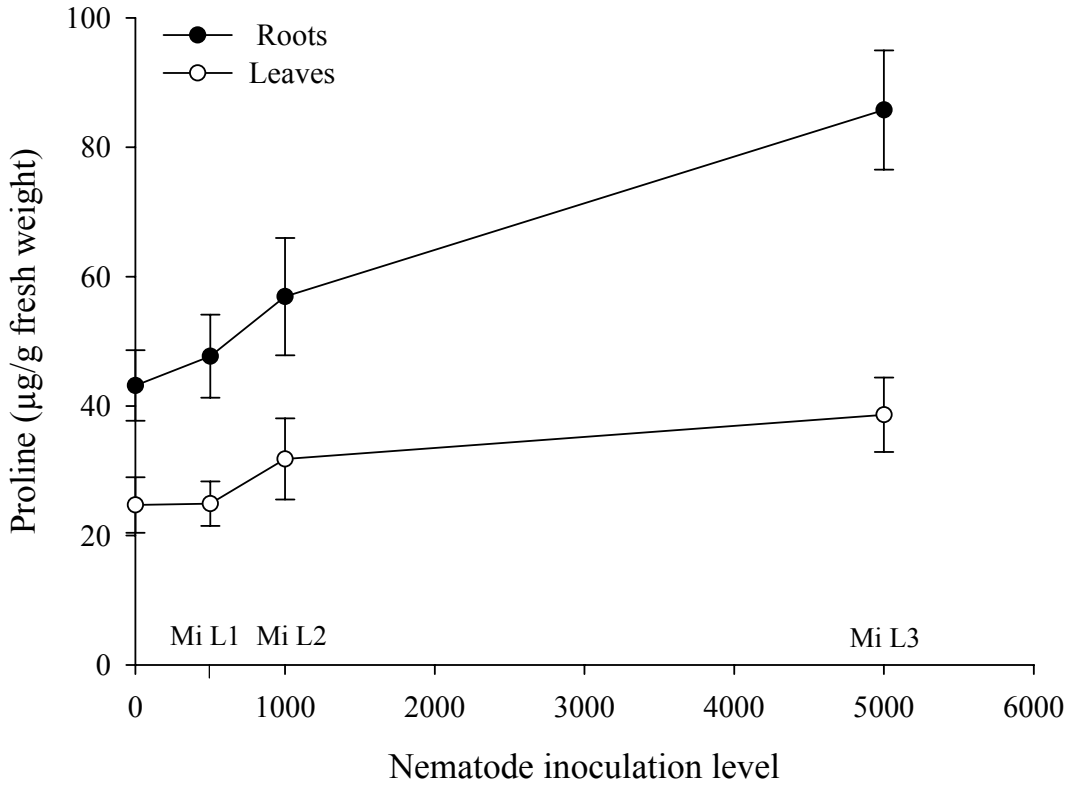


Figure 4.6: The relationship between nematode inoculation level and proline concentration (± SE) in roots and leaves of tomato plants at harvest; Mi L1, Mi L2, Mi L3 = 500, 1000, and 5000 J2 *M. incognita*, respectively, n = 6.

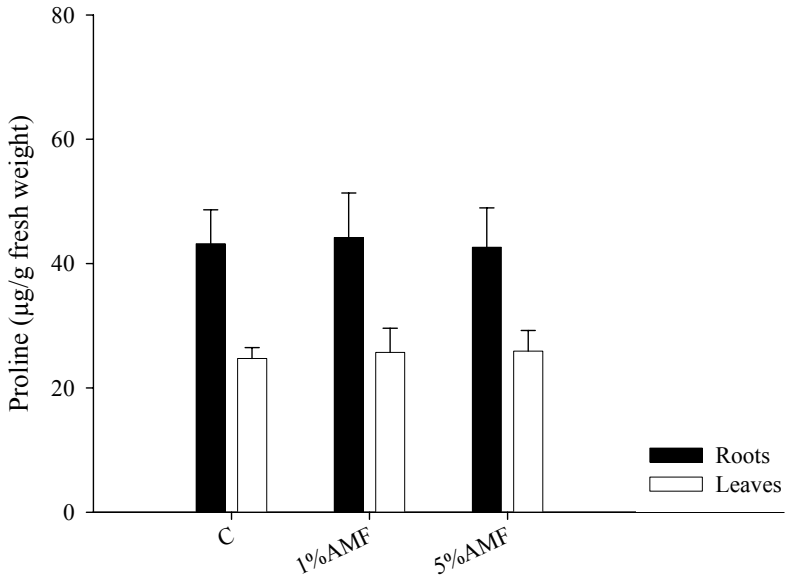


Figure 4.7: Effect of initial mycorrhizal inoculum density on proline concentration (±SE) in leaves and roots of tomato cv. King Kong II, C= non-inoculated, 1% AMF= inoculated with 1% v/v *G. intraradices* 510, 5% AMF= inoculated with 5% v/v *G. intraradices* 510, n = 6.

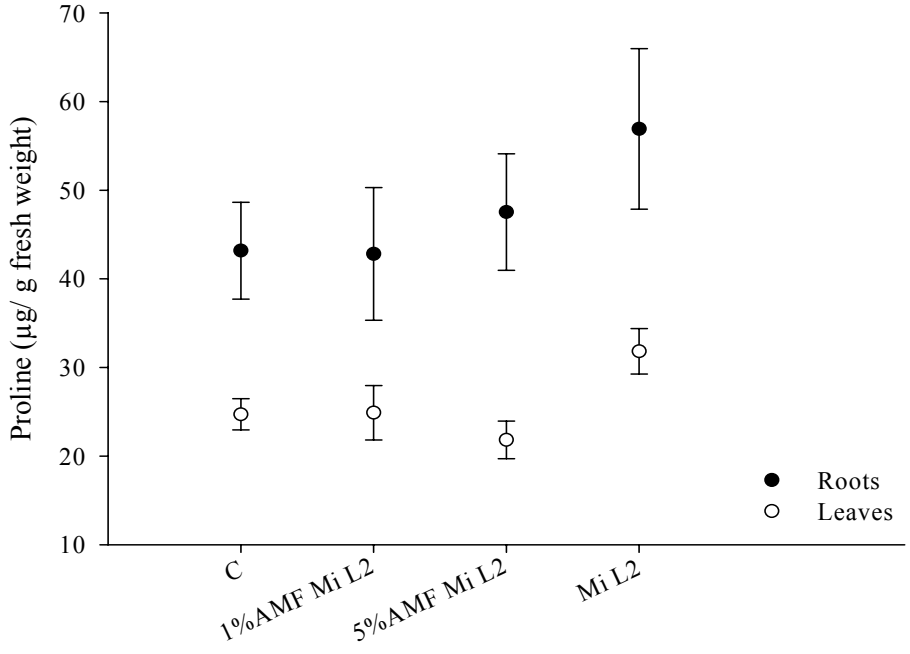


Figure 4.8: Effect of initial mycorrhizal inoculum density on proline concentration (±SE) in leaves and roots of tomato cv. King Kong II, C= non-inoculated, 1% AMF= inoculated with 1% v/v *G. intraradices* 510, 5% AMF= inoculated with 5% v/v *G. intraradices* 510, and L2= inoculated with 1000 J2 *M. incognita*, n = 6.

Inoculation with *M. incognita* resulted in a decrease of photosynthetic performance assessed as $PI_{abs}$. The results also show that this influence increased with increasing nematode inoculation levels and varied at different duration of infection (Figure 4.9, a). On the other hand, Figure 4.9, b exhibits a positive influence of both AMF inoculation levels on photosynthetic activity ($PI_{abs}$), however, this increase was observed only at earlier stages of the experiment and later diminished with time to a level equal to the non-inoculated control. The effect of AMF inoculation on the $PI_{abs}$ of nematode inoculated plants was also positive compared with the singly inoculated nematode treatment at one and three weeks after inoculation (Figure 4.9, c).

Figure 4.10 (a-c) demonstrates the results of the dissipated energy flux determined as dissipation per reaction centre (DI/RC). A comparison between nematode inoculated plants and the control exhibit a clear increase in DI/RC with increasing nematode inoculation level and duration of infection (Figure 4.10, a). AMF inoculation instead decreases dissipation of the healthy AMF treatments compared to the non-inoculated control (Figure 4.10, b), as well as in dual inoculation treatments with *M. incognita* compared to the singly inoculated *M. incognita* treatment (Figure 4.10, c).

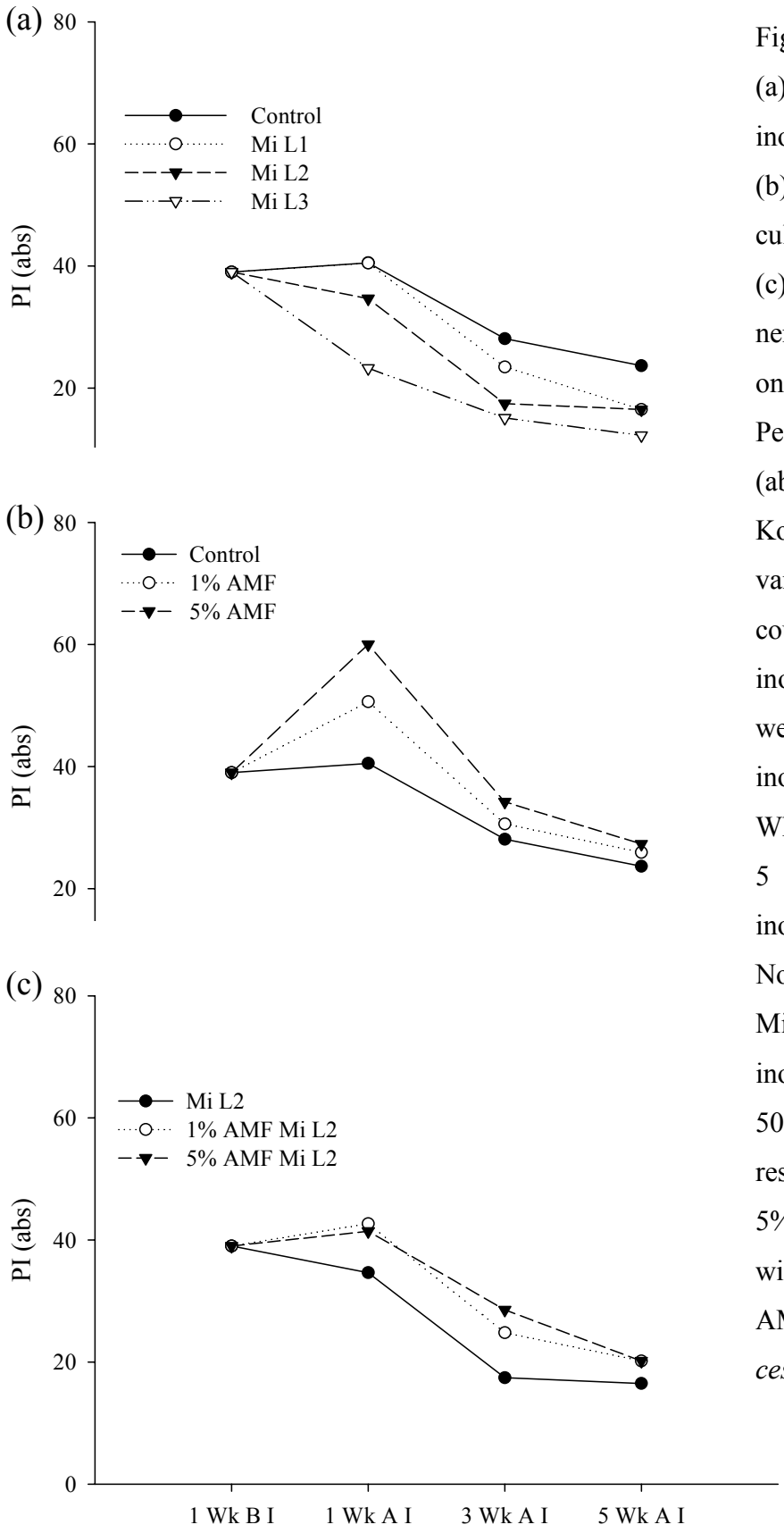


Figure 4.9: Effects of
(a) Different nematode inoculation levels,
(b) Different AMF inoculation densities and
(c) Combined AMF-nematode inoculation
on the vitality parameter Performance Index PI (abs) of tomato cv. King Kong II measured at various steps in the time course related to nematode inoculation (1Wk BI = one week before nematode inoculation, 1 Wk AI, 3 Wk AI, 5 Wk AI= 1,3, and 5 weeks after nematode inoculation respectively. Non-inoculated = Control. Mi L1, Mi L2, Mi L3 = inoculated with 500, 1000, 5000 J2 *M. incognita* respectively. 1% AMF, 5% AMF = inoculated with 1%, 5% v/v with the AM fungus *G. intraradices* 510.

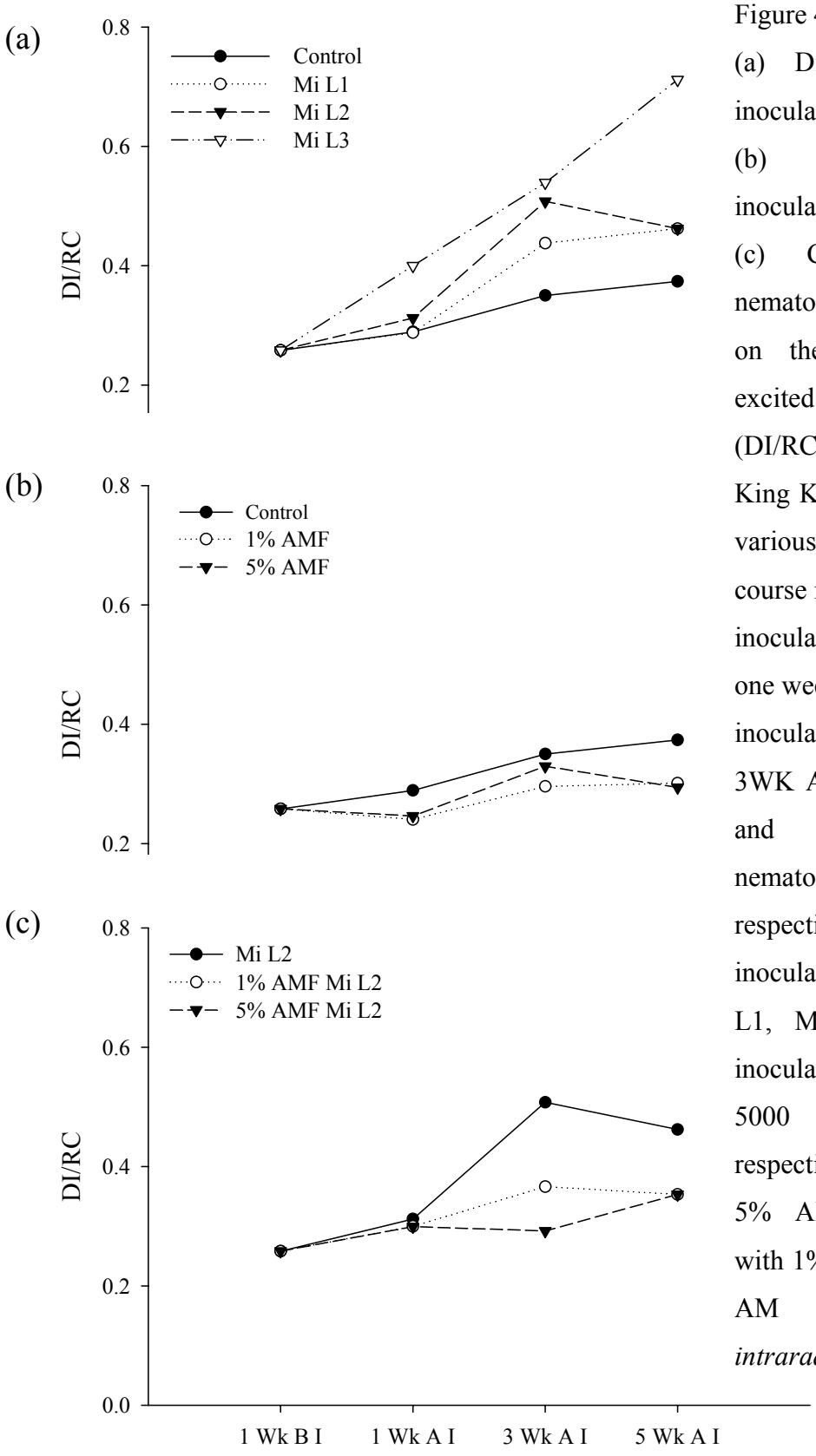


Figure 4.10: Effect of
(a) Different nematode inoculation levels,
(b) Different AMF inoculation levels and
(c) Combined AMF-nematode inoculation
on the dissipation per excited reaction centre (DI/RC) of tomato cv. King Kong II measured at various steps in the time course related to nematode inoculation (1WK BI = one week before nematode inoculation, 1WK AI, 3WK AI, 5 WK AI= 1,3, and 5 weeks after nematode inoculation respectively. Non-inoculated= Control, Mi L1, Mi L2, Mi L3 = inoculated with 500, 1000, 5000 J2 M. incognita respectively. 1% AMF , 5% AMF = inoculated with 1% , 5% v/v with the AM fungus *G. intraradices.*

### 4.5 Discussion

In the presented investigation, tomato plants inoculated with either level of the AM fungus *G. intraradices* 510 or *M. incognita* did not exhibit growth promotion or reduction due to the infection of either root colonizer. AMF improve plant growth through improved water and nutrient uptake by AMF external mycelium (Linderman *et al.*, 1994), enhanced synthesis of growth promoting hormones (Allen *et al.*, 1982) and increased number of vascular bundles. On the other hand, root-knot nematodes affect plant growth due to their consumption of host assimilates, through interfering with water and nutrient uptake by deforming roots thus diminishing and blocking water and nutrients absorption and translocation; nematode infection may also negatively influence the production of phytohormones (Melakeberhan and Webster, 1993). Abbot and Robson (1985) reported that inoculum density and potential play a role in influencing the degree of mycorrhizal dependency. Similarly, loss of biomass or yield due to nematode infection is a function of nematode inoculation level, nematode size, reproductive potential and duration of infection (Melakeberhan and Webster, 1993). The positive effects of either inoculation level of AMF and the negative influence of *M. incognita* on tomato growth in the presented study are obviously excluded by the good growth conditions in the greenhouse favouring rapid growth and nematode tolerance (Netscher and Sikora, 1990) and reducing mycorrhizal dependency. The results in this regard agree with those obtained by Talavera *et al.* (2001) and Diedhiou *et al.* (2003). The increase in root weight observed at the highest nematode inoculation level is likely attributed to nematode weight and the hypertrophic root tissue of the *M. incognita* galls.

The lower gall indices and numbers in nematode-AMF treatments confirm reports on the ability of AMF to suppress phytonematodes (Jaizme-Vega *et al.*, 1997; Parvatha Reddy *et al.*, 1998; Elsen *et al.*, 2001; Diedhiou *et al.*, 2003; Elsen *et al.*, 2003). The differences of the AMF treatments in colonizing roots were not translated into different degrees of nematode suppression. This clearly proves that the ability of AMF to prevent gall formation is not related to their ability to colonize roots. Nematode control appears when a certain level of mycorrhization is achieved. Saleh and Sikora (1984) reported that 38% AM colonization was required for *M. incognita* control on cotton by *G. fasciculatum*. Mycorrhization in both AMF treatments in the presented study exceeded this level; further increase in mycorrhization did not lead to a higher nematode control

which corroborates with the results obtained by Diedhiou *et al.* (2003) who tested the influence of *G. coronatum* against *M. incognita* on tomato. However, though increased mycorrhization - due to a higher initial inoculation level of AMF - did not result in increased nematode control, it reduced nematode gall size. Gall size is related to the number of nematodes in the tissue and differs among plant species in response to nematode infection (Hussey, 1985). Reduced gall sizes on mycorrhizal plants are attributed to either younger age of the galls, slower development of the female inside the gall, lower degree of hypertrophic development of the gall tissue and/or less nematodes in the tissue. Younger age of galls suggest less attractiveness of mycorrhizal roots and/or delayed penetration by the second stage juveniles (Oka *et al.*, 2000), while slower development of the female inside the gall is likely to be explained by competition for space and/or nutrients inside the roots. Moreover, it has been reported that ethylene production is correlated with increase in gall weight, suggesting its involvement in the hypertrophy of the tissues during gall formation (Glazer *et al.*, 1983). On the other hand, AM is reported to suppress ethylene production (Mc Arthur and Knowles, 1992). It could be that smaller galls on mycorrhizal plant are simply due to lower degree of hypertrophic development of the gall tissue. The exact mechanism causing the reduction in gall sizes in mycorrhizal plants is uncertain. The correlation between gall size and internal AMF colonization favours the idea of space competition or suppressed hypertrophic tissue. Moreover, the number of eggsacs produced on mycorrhizal and non-mycorrhizal plants was similar which in turn reflect similar number of mature females. However, a firm conclusion in this regard requires data on gall and female size of mycorrhizal and non-mycorrhizal treatments with synchronized J2 penetration. Smaller nematode sizes shall be in favour of plant health, since it is assumed that smaller nematodes have less energy demand and thus less influence on host growth (Melakeberhan and Webster, 1993).

Infection of plants with nematodes is associated with accumulation of amino acids (Stephan and Mc Clure, 1975; Mohanty and Pradhan, 1990; Hassan *et al.*, 1994; Sharma and Trivedi, 1996; Mohanty *et al.*, 1999). Amino acid content was reported to affect the degree of tolerance, resistance or susceptibility of tomato plants to nematodes (Lewis and Mc Clure, 1975; Showler *et al.*, 1991). Proline is the most rigid of the twenty naturally occurring amino acids. Its accumulation is a common response of plants to water deficit and salinity stress (Taylor, 1996); it was reported in several studies as one

of the most influenced amino acids by nematode infection (Sharma and Trivedi, 1996; Mohanty *et al.*, 1999). On the other hand, the possible influence of mycorrhizae on proline content in relation to environmental stress has been tested in several studies (Ruiz-Lozano *et al.*, 1995; Vivas *et al.*, 2003; Pinior *et al.*, submitted), mycorrhizal roots were reported to accumulate more proline than non-mycorrhizal ones, while the opposite was observed in shoots. The higher proline accumulation in mycorrhizal roots enhances osmotic adjustments contributing to maintenance of water potential gradients (Porcel and Ruiz-Lozano, 2004).

In tomato plants inoculated with *M. incognita*, proline concentration increased, although not significant, with an increase in nematode inoculum. Proline accumulation may be caused by water stress due to the disruption of xylem elements and reduced absorption and translocation of water and nutrients from roots to the shoot, due to the malformation of roots because of galling, or due to giant cells formation. Accumulation of proline due to water stress is a known phenomenon. However, plants were provided with adequate amounts of water through out the experiment, thus water stress could be excluded which is also supported by growth data. It is obvious that proline accumulation in this case is related to other factors than water stress. Moreover, plants subjected to water stress accumulate proline in shoots, whereas results obtained here exhibit higher proline content in roots compared to shoots. Meon *et al.* (1978) tested proline accumulation in roots and shoots of tomato infected by different levels of *M. javanica* in comparison to proline accumulation in cucumber (which does not accumulate proline under water stress, but did so due to *M. javanica* infection) and concluded that proline accumulation was induced by other factors than water stress. The authors proposed that the high metabolic activity in roots associated with giant cells and gall formation and with egg production (proline is a major constituent of nematode egg shells), exerts a requirement for energy which is supplied by free proline manufactured in the leaves and translocated to the site of nematode activity, a metabolic sink. However, the fact that proline concentration does not deceases in shoots while increases in the roots with increased nematode inoculation level and infection does not support the idea of the metabolic sink. Mohanty *et al.* (1999) attributed increased proline concentration in nematode infected roots compared with healthy ones to the breakdown of complex proteins during feeding process. This would explain the results obtained, since proline concentration increased with increasing nematode initial inoculum levels and infection. Moreover,

proline appears to be of critical importance in the formation of cell wall proteins, and the extensibility of the cell wall may be controlled by the amount of hydroxylation of the proline residues in it (Stephan and Mc Clure, 1975).

No clear relationship between proline and AMF colonization was observed. However, a trend of proline reduction in shoots and roots in mycorrhizal treatments was observed. How proline fits in the general pattern of the physiology of nematode-AMF interaction might be explained - if the results would be confirmed in further studies - in either of two ways: (1) proline is involved in formation of cell wall proteins and the extensibility of cell walls. Galls on mycorrhizal plants are reduced and giant cell systems reported to be smaller on mycorrhizal plants (Sikora, 1979), (2) On the other hand, if proline is to be considered an energy source for nematodes, it is then expected that less numbers of nematodes on mycorrhizal plant have lower energy demand.

Chlorophyll-*a*-fluorescence transients were analysed to quantify the behaviour of the photosystems in tomato plants inoculated with different levels and combinations of AMF and nematodes. This method has been used to screen decreases in photosynthetic activities caused by abiotic stress (e.g. Strasser *et al.*, 1996; Clarck *et al.*, 1998). Influence of inoculation with rhizobacteria and mycorrhizal fungi on photosynthetic activity of alfalfa was also tested (Tsimilli-Michael *et al.*, 2000) and stress buffer capacity of AMF measured on roses under drought (Pinior *et al.*, submitted) and on *Pisum sativum* plants suffering from cadmium stress (Rivera-Becerril *et al.*, 2002). Briefly, this method depends on calculations based on the fluorescence rise exhibited during the first second of illumination of a photosynthetic material which shows a sequence of phases from the initial to the maximum fluorescence values and have been labelled O, J, I, P (Strasser and Govindjee, 1992). The shape of the O, J, I, P transient is influenced by several environmental factors. Analysis of the O, J, I, P transients (named "JIP-test") leads to the calculation of structural and functional parameters to quantify the behaviour of photosystem II (Strasser and Strasser, 1995). Measurements using this method are carried out quickly and thus can be applied easily for screening many samples; moreover, the method is non-invasive.

The vitality of plants inoculated by different levels and combinations of AMF and nematodes was characterized with the Performance Index which accounts for the

functionality of photosystems I and II. The effect of any microbial inoculation is a result of complex interactions with the plant. Both AMF and nematodes are reported to influence photosynthetic rate in host plants. *M. incognita* infection negatively influenced the performance index $PI_{abs}$ and this impairment increased with raised nematode inoculum density. From the results obtained it seems that at least one component of the $PI_{abs}$ parameter (TR/ABS, ET/TR, ET/ABS) is influenced by nematode infection. Melakeberhan *et al.* (1991) used the chlorophyll-*a*-fluorescence technique to assay the activity of water splitting complex of PSII and showed a decline in its activity within 24 hours after nematode (*Bursaphelenchus xylophilus*) inoculation. The reduction of the water splitting complex of PSII results in a reduced number of electrons (splitting one $H_2O$ molecule = 2 $H^+$ + ½ $O_2$ + 2 *é*) in the electron transport chain, which in turn reduces the probability that an absorbed photon will move an electron into the electron transport chain. If nematode infection is positively correlated with the reduction level of the water splitting complex of PSII, then reduction in $PI_{abs}$ is explained also with this trait. Results of the dissipation per reaction centre (DI/RC), reflecting non-photochemical processes like heat or chlorophyll-fluorescence (Govindjee 1995) are in accordance with those of $PI_{abs}$. Nematode infection results in a higher DI/RC compared to the healthy control plants, and as nematode infection increases, this leads to a less active photosynthetic state. The observed reduction in $PI_{abs}$ and increase in DI/RC with the course of time during the experiment could be attributed to physiological senescence of leaves.

On the other hand, plants inoculated with AMF exhibited a higher performance index $PI_{abs}$ compared to the control. The results corroborate with those obtained by Tsimilli-Micheal *et al.*, (2000), who found through evaluating extra parameters in the JIP-test that AMF increases electron transport activity per leaf area. Combined AMF-nematode inoculation resulted also in increased values for $PI_{abs}$ and decreased values for DI/RC compared to the singly inoculated nematode treatment. This reflects a better vitality in mycorrhizal plants even when this is not reflected directly on plant biomass production.

The results obtained demonstrate that the different inoculation levels and combinations of *G. intraradices* - *M. incognita* exhibit differences when analysed by means of the JIP-test. However, due to the lack of studies using different microbial combinations, the overall significance of the results obtained cannot be estimated yet.

# 5 Dual inoculation of AMF and rhizobacteria for the improvement of biocontrol of *Meloidogyne ingognita* on tomato

**Abstract**

Inoculation of tomato (cv. King Kong II) with the AM fungus *G. intraradices* 510 or either rhizobacteria; *Cellulomonas turbata* (SR1) or *Acinetobacter baumannii* (SR6) did not enhance growth of the tomato plants. Single inoculation of either bacterium did not suppress *M. incognita* infection. *G. intraradices* suppressed nematodes infection and development. The concomitant inoculation of the AM fungus and either bacterial strain enhanced the suppression of nematode galls and eggsacs. When concomitantly inoculated, the mycorrhizal symbiosis did not influence the bacterial population density. Co-inoculation of SR1 or SR6 with *G. intraradices* did not enhance mycorrhizal colonization.

## 5.1 Introduction

Biological control of plant parasitic nematodes is often regarded as a non-acceptable alternative for pesticides. Reasons behind this include: inconsistent performance, low efficacy and slower action when compared to pesticides (Meyer and Roberts, 2002). One approach to improve antagonists' performance is to include multiple biocontrol agents in the nematodes control strategies (Sikora, 1992). Interactions between two organisms may produce completely different effects on plants than the separate effects of each and even the sum of their separate effects. Potential advantages of bio-control agents applied in combination include: (i) multiple modes of action against the target pathogen or nematode, (ii) ability to affect more than one stage of the life cycle of the target organism, (iii) activity of microbes during different times in the growing season, (iv) increased consistency in performance over a wider range of soil conditions, and (v) potential to select organisms that affect more than one plant pathogen (Meyer and Roberts, 2002).

Rhizobacteria are part of the natural microflora of healthy plants; they may be important contributors to plant health and general soil suppressivness (Kloepper *et al.*, 1999). Bacteria that possess antagonistic features against pathogens or produce compounds that stimulate plant growth are called plant health promoting rhizobacteria (PHPR) or plant growth promoting rhizobacteria (PGPR) (Sikora, 1992; Kloepper *et al.*, 1999). Many

studies demonstrated the ability of some rhizobacteria strains to suppress a variety of soil-borne pathogens (Oostendorp and Sikora, 1990; Raupach and Kloepper, 1998; Hoffmann-Hergarten *et al.*, 1998; Reitz *et al.*, 2001; Jetiyanon *et al.*, 2003; Siddiqui and Shaukat, 2003). Although a wide range of bacterial genera and species were studied, the typical representatives of PGPR are the members of the genera *Pseudomonas* and *Bacillus*. PGPR antagonize soil pathogens by competing for resources such as iron, by production of antibiotics or enzymes, or by inducing systemic resistance in plants (van Loon *et al.*, 1998).

Apart from influencing plant growth and health, some rhizobacteria may interact and influence the growth and function of other soil microflora. Among the diverse interactions between rhizobacteria and other soil micro-organisms are those with AMF. In recent years, several types of bacteria have been reported to be associated with the rhizosphere of plants colonized by AMF and have been identified as N2 fixing bacteria, plant growth promoting rhizobacteria, phosphate solubilizing bacteria and antagonists of plant pathogens (Budi *et al.*, 1999). It has been demonstrated that some rhizobacteria have the ability to influence mycorrhizal colonization (von Alten *et al.*, 1993; Garbaye, 1994; Gryndler and Vosatka, 1996); those have been named mycorrhiza helper bacteria (MHB) (Garbaye, 1994). von Alten *et al.* (1993) reported stimulation of mycorrhizal development in eight different crops when inoculating the plants with both *G. intraradices* and the rhizobacterium *Bacillus mycoides*. Similarly, Duponnois and Plenchette (2003) described the ability of the *Pseudomonas monteilii* strain HR13 to promote the colonization of several Australian *Acacia* species by ectomycorrhizal fungi and one endomycorrhizal fungus. The combined inoculation enhanced growth of several species compared to single inoculation treatments. Among the proposed mechanisms underlying the effect of MHB are (i) stimulation of AMF hyphal growth in the rhizosphere, (ii) increase of the receptivity of the root, (iii) production of phenolic compounds such as hypaphorine, and (iv) increase of the aggressiveness of the fungal hyphae (Garbaye, 1994; Duponnois and Plenchette, 2003).

Compared with the substantial volume of work reported on the single use of rhizobacteria, AMF, and other antagonists to control plant parasitic nematodes, considerably less work has been done to examine the potential of combined inoculants. Management of multi-microbial interactions could be a promising control approach;

however, candidate biocontrol agents to be used in combinations must be compatible. Arising from the believe that associated organisms may complement mycorrhizal activities, increased research is being conducted using combined inoculations of AMF and bacteria.

Two rhizobacteria, *C. turbata* (SR1) and *A. baumannii* (SR6) - both isolated from AMF spores - were selected because of their antagonistic behaviour against RKN. Both strains were effective in suppressing *M. incognita* infection in tomato (Reimann and Sikora, 2003).

Bacteria that belong to the genus *Acinetobacter* are Gram-negative, strictly aerobic, are found in soil and water, and considered as opportunist pathogens in humans. In contrast, members of the genus *Cellulomonas* are Gram-positive, aerobic or facultatively anaerobic, and found mostly in soil (Singleton, 1999).

The main objectives of this study were to determine the effects of the two rhizobacteria SR1 and SR6 i) on the control of *M. incognita*, ii) on mycorrhizal colonization by *G. intraradices*, and iii) the possible stimulation of the mycorrhizal effect against *M. incognita*.

**5.2 Material and methods:**

The tomato (*L. esculentum* Mill.) cultivar King Kong II (Known-You Seed Co. Ltd., Taiwan) was used.

**5.2.1 Bacteria**

Bacterial cultures were maintained on bouillon agar medium (BN).

| | | |
|---|---|---|
| Bouillon agar: | meat extract | 10 g |
| | pepton | 10 g |
| | NaCl | 5 g |
| | agar | 18 g |
| | a. dest. | ad 1000 ml |

#### 5.2.1.1 Selection of marker strains

SR1** and SR6 ** are spontaneous rifampicin and streptomycin resistant derivatives of the wild type strains of SR1 and SR6, respectively; they were selected as tools for monitoring the population density of the bacteria throughout the experimental period. The bacterial strains were sub cultured on BN plates and incubated for 48 h at 28°C. A selected single colony was transferred into 3 ml CASO broth (CB, 30 g/l, Fluka, Germany) and incubated on a rotary shaker (110 rpm) at 28°C. After 24 h, 2 ml of the turbid bacterial culture were inoculated into 200 ml CB in a 500 ml Erlenmeyer flask and incubated until the suspension became turbid. Then 25 ppm (final concentration) sterile filtered rifampicin solution was added and the bacteria were reincubated for another 24 h. Two ml of the bacterial suspension were then added to 200 ml CB containing 25 ppm rifampicin and incubated until turbidity. After two days the same procedure was repeated with 50 ppm rifampicin, and after another two days with 100 ppm rifampicin to obtain mutants resistant to 100 ppm rifampicin.

In order to obtain mutants resistant against two antibiotics (rifampicin and streptomycin), 2 ml of the bacterial culture containing 100 ppm rifampicin were transferred into 200 ml CB with 25 ppm rifampicin and 25 ppm sterile filtered streptomycin and incubated for 2 days in the shaker. The same procedures to obtain the mutant resistant against rifampicin were followed to obtain the mutants resistant for both antibiotics. Bacteria surviving the 100 ppm rifampicin and 100 ppm streptomycin in the liquid culture were further subjected three times to single-colony isolation on rifampicin and streptomycin (100 ppm of each) supplemented BN agar to test for stability (Sikirou, 1999).

#### 5.2.1.2 Bacteria inoculation

For inoculation, a single colony of each bacterial isolate was cultured in 100 ml of CB in 250-ml Erlenmeyer flasks incubated on a rotary shaker for 20 h (100 rpm, 28°C in the dark). When the antibiotic resistant mutants were used, the medium used for culturing them was supplemented with 100 µl/l streptomycin and rifampicin. Bacterial suspensions were adjusted to an optical density of 2 at 650 nm, and cells were then collected by centrifugation (5000 $g_{av}$, 20 min, 10°C) the pellet was washed twice with, and re-suspended in sterile ¼ Ringer's solution.

The inoculum concentrations were estimated by dilution plating; which corresponded to a concentration of $2.6\times10^5$ cfu/ml and $4.4\times10^5$ cfu/ml for SR1 and SR6 respectively. Inoculation of tomato plants was performed by pipetting 5 ml bacterial suspension around the stem base.

#### 5.2.1.3 Re-isolation of bacteria from rhizosphere and population assessment

At the end of the experiment, pots were entirely colonized by the root system, and it was assumed that the measurements were made on rhizosphere soil. The abundance of the bacteria in the rhizosphere at different times during the experiment was determined by dilution plating from the rhizosphere. Three plants were randomly chosen, substrate samples were taken with a cork borer from each pot and soil suspensions (1:10) were prepared with sterile distilled water and then serially diluted. 100 µl aliquots were plated on BN agar supplemented with rifampicin and streptomicin (100 µg/ml). After incubation for 48 h at 28°C, the number of cfu was determined and expressed per gram of soil dry weight. Re-isolation of the bacteria from the rhizosphere took place three times: at nematode inoculation, at the second date of bacterial inoculation, and at harvest (Figure 5.1).

### 5.3 Experimental set-ups

Newly germinated tomato plants were transferred to Jiffy pots into a mixture of sterile sand and commercial compost (3:1), containing, according to the treatment, 10% (v/v) of mycorrhizal inoculum. Non-mycorrhizal treatments received the same amount of expanded clay. Bacteria were inoculated twice during the experimental period: the first inoculation took place when AMF colonization achieved 20% (3- to 4- leaf stage; 14 to 18 days after sowing), 5 ml of bacterial inoculum were applied in the Jiffy pot around the stem base, non-bacterial treatments received 5 ml Ringer's solution. One day later, seedlings were transplanted into 800 ml plastic pots. One week after transplanting, when the seedlings developed root system, each plant was inoculated with 500 *M. incognita* juveniles. The second bacterial inoculation was done two weeks after the first bacterial inoculation (i.e. one week after nematode inoculation).

The experiment was conducted twice. The first experiment was conducted during the summer months (May-July), plants were then fertilized twice a week (Experiment I). The repetition was conducted during winter months (January-March) and included the

antibiotic resistant strains to monitor the population density of the bacteria (Experiment II). Plants of the repetition experiment were fertilized once a week. Harvest took place 5 weeks after potting. Shoot and root weight were recorded, the numbers of galls induced by *M. incognita* were counted using a stereomicroscope, and mycorrhization parameters (colonization frequencies and intensities) were rated.

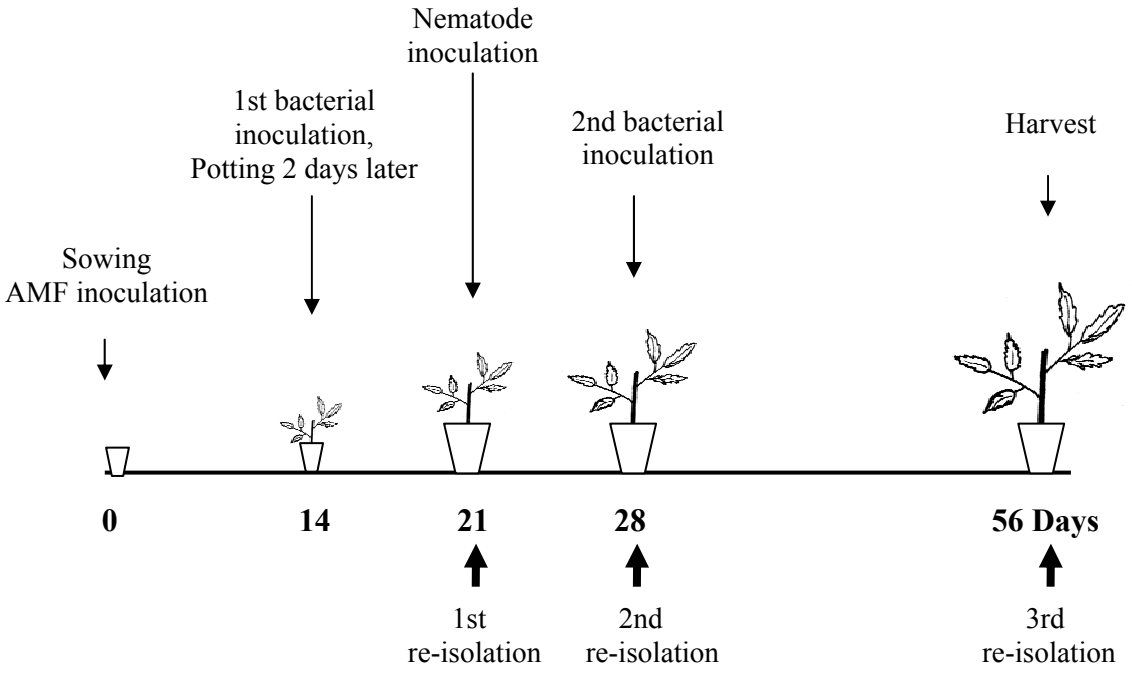


Figure 5.1: Schematic overview of the time frames used in the AMF-rhizobacteria combination experiment.

Experiment I consisted of 8 treatments:

1. control (C),
2. *M. incognita* (Mi),
3. *G. intraradices* 510 (AMF),
4. *G. intraradices* 510 + *M. incognita* (AMF + Mi),
5. SR1 + *M. incognita* (SR1 + Mi),
6. SR6 + *M. incognita* (SR6 + Mi),
7. *G. intraradices* 510 + SR1 + *M. incognita* (AMF SR1 +Mi),
8. *G. intraradices* 510 + SR6 + *M. incognita* (AMF SR6 + Mi).

Each treatment consisted of 6 replicates.

Experiment II consisted of 12 treatments:

1. Control (C),
2. *M. incognita* (Mi),
3. *G. intraradices* 510 (AMF),
4. *G. intraradices* 510 + *M. incognita* (AMF + Mi),
5. SR1 + *M. incognita* (SR1 + Mi),
6. SR1** + *M. incognita* (SR1** + Mi ),
7. SR6 + *M. incognita* (SR6 + Mi),
8. SR6** + *M. incognita* (SR6** + Mi ),
9. *G. intraradices* 510 + SR1 + *M. incognita* (AMF SR1 + Mi),
10. *G. intraradices* 510 + SR1** + *M. incognita* (AMF SR1** + Mi),
11. *G. intraradices* 510 + SR6 + *M. incognita* (AMF SR6 + Mi),
12. *G. intraradices* 510 + SR6** + *M. incognita* (AMF SR6** + Mi).

Each treatment consisted of 8 replicates.

The treatments of both experiments were laid out in a completely randomised design.

**5.4 Results**

Data obtained in experiments I and II show that neither treatment had an effect on plant growth parameters (Tables 5.1 and 5.2). Shoot and root weights of plants in Experiment I were higher than those obtained in Experiment II. The colonisation by the AM fungus was not influenced by the co-inoculation with either bacterium or its antibiotic resistant derivative, and neither by *M. incognita*. Levels of AMF colonization were similar in both experiments.

Table 5.1: Shoot fresh weight (Sh FWT), root fresh weight (R FWT), and mycorrhizal infection (MI) parameters (MF, % colonized root pieces of 1 cm length and MD, density of fungal particles in the roots) (±SE) of plants used in Experiment I, AMF= *G. intraradices* 510, SR1= *C. turbata*, SR6= *A. baumannii*, Mi = *M. incognita*, n = 6.

| Treatment | Sh FWT [g] | R FWT [g] | MI | |
|---|---|---|---|---|
| | | | MF | MD |
| **Control** | 70.0 ± 3.0 | 27.8 ± 1.0 | | |
| **Mi** | 66.9 ± 2.4 | 27.3 ± 1.8 | | |
| **AMF** | 70.2 ± 2.4 | 33.8 ± 2.4 | 40.0 ± 9.9 | 1.1 ± 0.1 |
| **AMF + Mi** | 68.5 ± 1.2 | 33.1 ± 1.6 | 40.8 ± 12.0 | 1.4 ± 0.1 |
| **SR6 + Mi** | 69.9 ± 1.9 | 35.4 ± 2.2 | | |
| **AMF SR6 + Mi** | 74.6 ± 2.3 | 32.3 ± 1.3 | 42.5 ± 7.0 | 1.2 ± 0.1 |
| **SR1 + Mi** | 70.6 ± 1.6 | 33.0 ± 1.3 | | |
| **AMF SR1 + Mi** | 71.5 ± 2.7 | 31.0 ± 2.4 | 49.2 ± 9.2 | 1.2 ± 0.1 |

Table 5.2: Shoot fresh weight (Sh FWT), shoot dry weight (Sh DWT), root fresh weight (R FWT), and mycorrhizal infection (MI) parameters (MF, % colonized root pieces of 1 cm length and MD, density of fungal particles) (± SE) of plants used in Experiment II. AMF = *G. intraradices* 510, SR1= *C. turbata*, SR6= *A. baumannii*, Mi = *M. incognita*, SR1**, SR6** are the antibiotic mutants of SR1 and SR6 respectively, n = 8.

| Treatment | Sh FWT [g] | Sh DWT [g] | R FWT [g] | MI | |
|---|---|---|---|---|---|
| | | | | MF | MD |
| **Control** | 34.5 ± 0.6 | 6.7 ± 0.2 | 8.0 ± 0.4 | | |
| **Mi** | 33.2 ± 0.9 | 6.3 ± 0.2 | 9.0 ± 0.6 | | |
| **AMF** | 32.3 ± 0.7 | 6.1 ± 0.2 | 7.8 ± 0.5 | 41.3 ± 3.2 | 1.1 ± 0.05 |
| **AMF + Mi** | 32.2 ± 0.9 | 6.2 ± 0.2 | 8.6 ± 0.5 | 38.7 ± 2.5 | 1.2 ± 0.03 |
| **SR6 + Mi** | 35.9 ± 0.5 | 7.0 ± 0.1 | 9.8 ± 0.5 | | |
| **AMF SR6 + Mi** | 34.9 ± 0.9 | 6.6 ± 0.2 | 9.1 ± 0.3 | 48.8 ± 4.2 | 1.2 ± 0.05 |
| **SR6$^{**}$ + Mi** | 37.3 ± 0.5 | 7.2 ± 0.2 | 8.5 ± 0.4 | | |
| **AMF SR6$^{**}$ + Mi** | 34.9 ± 0.6 | 6.7 ± 0.2 | 7.7 ± 0.3 | 45.6 ± 4.5 | 1.2 ± 0.02 |
| **SR1 + Mi** | 32.7 ± 0.7 | 6.2 ± 0.2 | 8.2 ± 0.5 | | |
| **AMF SR1 + Mi** | 36.2 ± 0.7 | 6.9 ± 0.1 | 8.1 ± 0.3 | 48.8 ± 3.1 | 1.2 ± 0.04 |
| **SR1$^{**}$ + Mi** | 34.0 ± 0.8 | 6.9 ± 0.2 | 8.3 ± 0.6 | | |
| **AMF SR1$^{**}$ + Mi** | 35.9 ± 0.9 | 7.1 ± 0.3 | 7.3 ± 0.4 | 47.5 ± 2.7 | 1.1 ± 0.03 |

The results obtained in Experiment I show that the single inoculation of the AM fungus *G. intraradices* 510 and both bacterial isolates SR1 and SR6 did not influence the number of galls compared to the *M. incognita* treatment (Figure 5.2). However, a significant reduction in gall numbers was observed in plants inoculated concomitantly with both the AM fungus and either bacterial isolates. When inoculated concomitantly with the mycorrhizal fungus both bacterial isolates suppressed nematode galls. Both bacteria were equally effective in co-inoculation treatments.

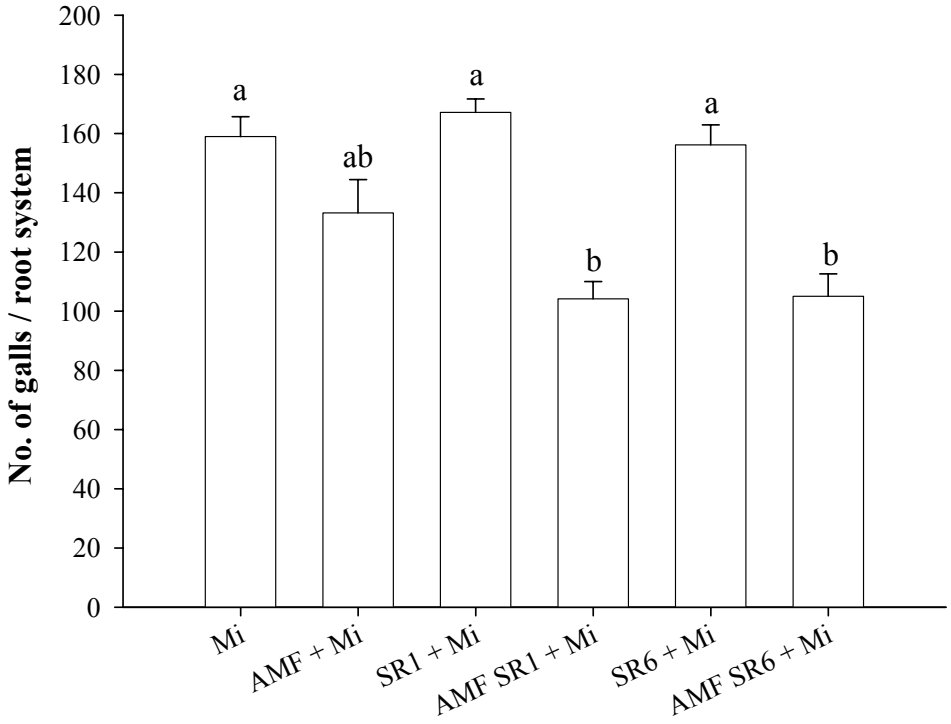


Figure 5.2: Number of galls per root system (± SE) induced by *M. incognita* in AMF-rhizobacteria combination experiment I. Treatments were: *M. incognita* (Mi), *G. intraradices* 510 + *M. incognita* (AMF + Mi), SR1 + *M. incognita* (SR1 + Mi), SR6 + *M. incognita* (SR6 + Mi), *G. intraradices* 510 + SR1 + Mi (AMF SR1+ Mi), *G. intraradices* 510 + SR6 + Mi (AMF SR6 +Mi). Bars followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$), n = 6.

Results obtained from Experiment II exhibit the same tendency of the results obtained in Experiment I. The AMF treatment significantly reduced number of galls compared to the *M. incognita* treatment. The single inoculation of the bacterial strains or their

antibiotic resistant derivatives had no influence on the number of galls. All concomitant inoculation treatments reduced the number of galls compared to the *M. incognita* and also to the AMF treatments. Effects on nematode galls were almost doubled compared to the singly AMF treatment. There were no differences in the level of gall reduction among the concomitant inoculation treatments (Figure 5.3, a). With regards to the number of eggsacs, the AMF treatment and all concomitant inoculation treatments reduced eggsacs when compared to the *M. incognita* treatment and all singly inoculated bacteria treatments except the SR1 treatment (Figure 5.3, b). Differences in gall sizes were not observed in this experiment (data not shown).

Over the whole experimental period concomitant inoculation with the AM fungus had no significant influence neither on the density of the bacterial population, nor on the rhizosphere colonization pattern of either bacterium (Figure 5.4). However, when treatments were compared over the three sampling periods, an increase in the population density of both rhizobacteria was observed at harvest. Treatments inoculated with *A. baumannii* had a higher number of cfu per g at harvest than treatments inoculated with *C. turbata*.

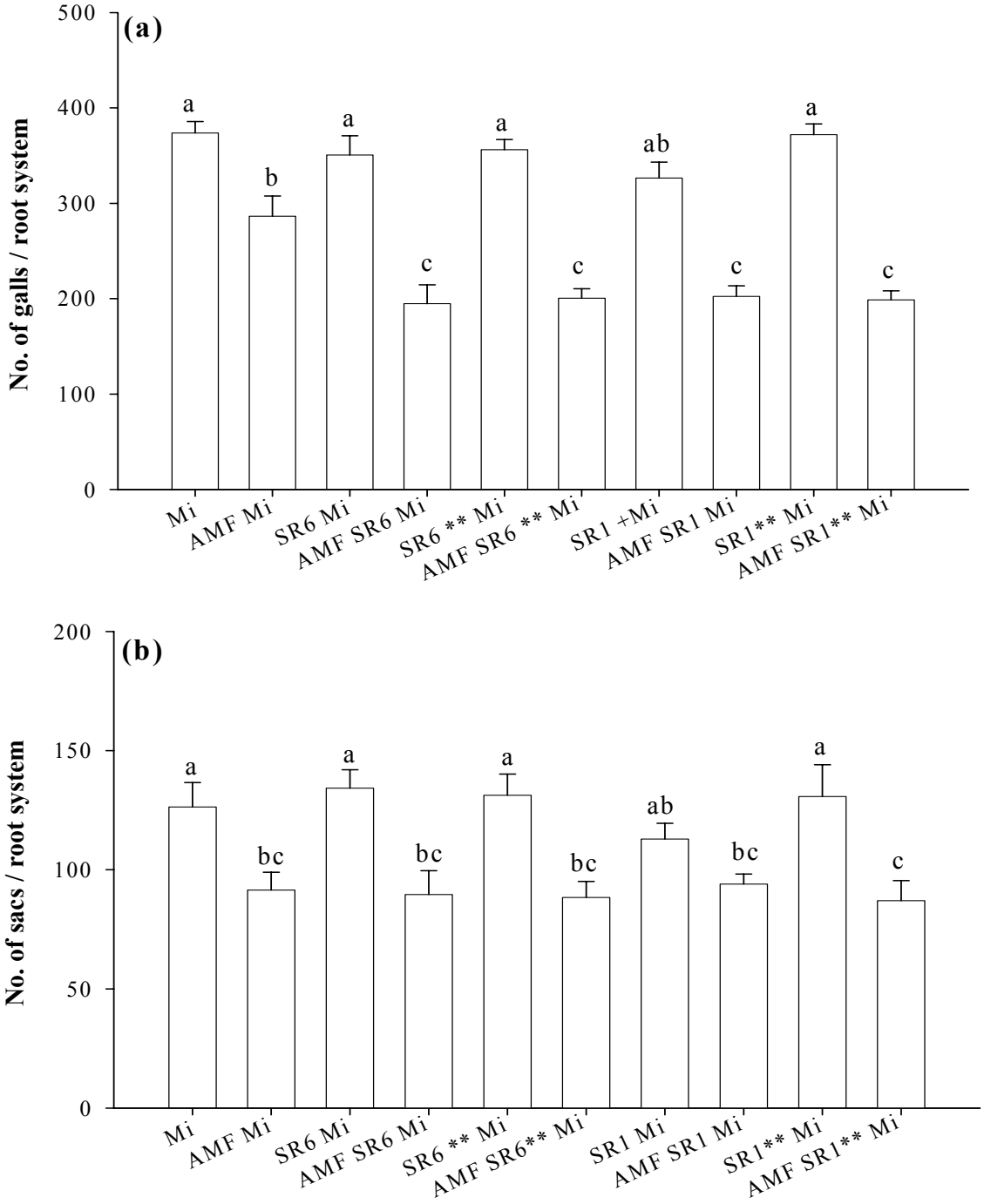


Figure 5.3: Number of (a) galls & (b) eggsacs per root system (±SE) induced by *M. incognita* experiment II. Treatments were: *M. incognita* (Mi), *G. intraradices* 510 + *M. incognita* (AMF Mi), SR1 + *M. incognita* (SR1Mi), SR1$^{**}$ + *M. incognita* (SR1$^{**}$ Mi), SR6 + *M. incognita* (SR6 Mi), SR6$^{**}$ + *M. incognita* (SR6$^{**}$ Mi), *G. intraradices* 510 + SR1 + *M. incognita* (AMF SR1 Mi), *G. intraradices* 510 + SR6 + *M. incognita* (AMF SR6 Mi), *G. intraradices* 510 + SR1$^{**}$ + *M. incognita* (AMF SR1$^{**}$ Mi), *G. intraradices* 510 + SR6$^{**}$ + *M. incognita* (AMF SR6$^{**}$ Mi). Bars followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$), n = 8.

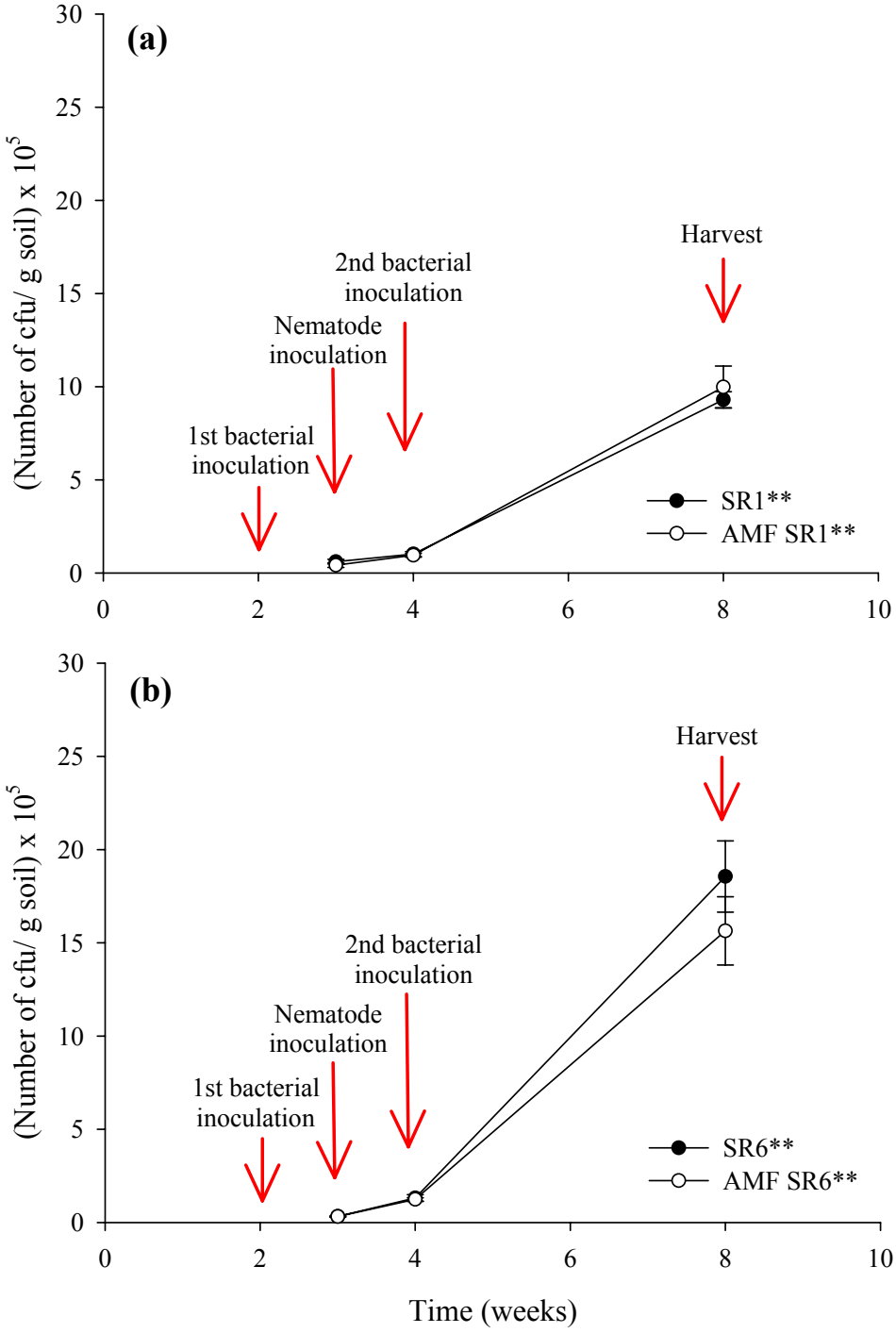


Figure 5.4: Colony forming units (cfu) per g soil by the rhizobacteria *C. turbata* (a) and *A. baumannii* (b), isolated from soil infested with *M. incognita*, inoculated and non-inoculated with the AM fungus *G. intraradices* 510 (a) shows the rhizosphere colonization pattern by *C. turbata* when inoculated singly (SR1**) and concomitantly with *G. intraradices* 510 (AMF SR1**), (b) shows the rhizosphere colonization pattern by the *A. baumannii* when inoculated singly (SR6**) and concomitantly with *G. intraradices* 510 (AMF SR6**), n = 3.

**5.5 Discussion**

Neither treatment had growth promotion influence in both experiments. The lack of growth response might be due to optimum growth conditions in the greenhouse which favour rapid growth and tolerance to nematode attack (Netscher and Sikora, 1990). The better growth of plants in Experiment I is likely attributed to differences in the dominant environmental conditions and to the higher fertilization during the experiment in summer. In spite of the controlled growth environment in the greenhouse, external environmental factors exert major influences that may demand different cultural practices -such as watering and fertilization- during the experiments conducted at different seasons. The difference in nematode inoculum efficiency (number of J2 inoculated: the number of nematodes that developed galls) between the two experiments can be therefore attributed to either enhanced plant tolerance or to direct influence of the prevailing environmental conditions on the J2 inoculum. The first experiment was conducted in the summer; the nematode juveniles may have suffered difficult penetration condition due to lower moisture content.

With regard to the influence on nematode infection, the results obtained show that both SR1 and SR6 did not control *M. incognita* although previously shown to have biological control activity. In a study comparing nine bacteria isolated from the mycorrhizosphere Reimann and Sikora (2003) detected significantly less number of *M. incognita* penetrating roots treated with either SR1 or SR6 three weeks after nematode inoculation. Variation in the biocontrol activities of rhizobacteria is a known phenomenon (Weller, 1988). Abiotic and biotic factors, as well as delivery methods and rates may play major roles. Substrate has a major influence on bacterial communities in relation to nematode control; density and diversity are influenced by level of organic amendments (Hallmann et al., 1999)

The absence of effect of the singly inoculated bacterial treatments and the identical population densities in mycorrhizal and non-mycorrhizal rhizosphere exclude the possibility of direct influence of either bacterium or its metabolites on activity or infectivity of J2. Thus detail tests were not considered necessary. Modes of actions by which bacteria suppress plant pathogens include growth promotion, induction of systemic resistance, competing for resources such as iron and production of antibiotics and lytic enzymes (van loon, 1998).

The main results of the study suggest that the co-inoculation of tomato with the AM fungus *G. intraradices* 510 and either bacterium SR1or SR6 results in a synergism against *M. incoognita* and improve the efficacy of *M. incognita* control conferred by the single inoculation of the AM fungus.).

The better performance of the dual treatment in suppressing nematode infection may be due to specific attributes of micro-organisms under the described experimental system. Nematode infection can be suppressed with short-term disruption of early root penetration (Sikora and Hoffman-Hergarten, 1993).

The ability of the tested bacteria to stimulate mycorrhizal development and function, as well as mycorrhizal effects on the associated bacterial population density and development do not seem to play a role. Certain bacteria are reported to stimulate mycorrhizal formation and development (von Alten *et al.*, 1993; Garbaye, 1994). However, comparing mycorrhizal colonization in singly inoculated AMF treatments with dually inoculated treatments revealed no differences in either mycorrhizal colonization parameters (frequency and intensity). It was reported that some bacteria are capable to accelerate mycorrhizal colonization at early stages and that such influence diminishes with time (von Alten *et al.*, 1993). Therefore bacterial inoculation may have had accelerated the mycorrhizal colonization during early phases of the interaction process. However, bacteria were inoculated when AMF achieved colonization frequency of 20%, and this developed into approximately 49% after six weeks in the best case, which does not favour the idea of accelerated colonization. Detailed estimation of the AMF colonization throughout the whole experimental period and testing the influence of bacterial inoculation timing may reveal the exact influence of the co-inoculation with either bacterium. Moreover, AM colonization was quantified by assessing the level of fungal infection in plant roots after staining which is not a proper indicator for the functional aspect of symbiosis (Vierheilig and Ocampo, 1989). If the enhanced nematode suppression in dual inoculation treatments is due to stimulated functional aspects of the symbiosis and the two tested bacteria are to be considered as MHB, the main proposed mechanisms for the MHB effect are to stimulate hyphal growth in the rhizosphere (Duponnois and Plenchette, 2003). Among other mechanisms, Garbaye (1994) proposed possible effects of MHB on the receptivity of the root, on

stimulation of the production of phenolic compounds such as hypaphorine, and on increased aggressiveness of the fungal symbiont.

However, the above mentioned parameters were not evaluated in this study. The two tested bacteria exhibited similar levels of compatibility in co-inoculation with AMF, might be because they are originally isolated from a related microbial community. Reports regarding MHB specificity are controversial; while Garbaye and Duponnois (1992) demonstrated that the MHB effect was fungus specific, these findings were not supported by other studies (Duponnois and Plenchette, 2003).

The results of the bacterial re-isolation clarify that dual inoculation treatments did not exhibit higher bacterial population densities in the substrate. Therefore, the better *M. incognita* suppression was not due to better bacterial colonization of the rhizosphere in mycorrhizal plants. However, the identical bacterial colonization rates of the rhizosphere of the mycorrhizal and non-mycorrhizal plants do not necessarily reflect similar colonization and development of each bacterial isolate in the endorhizosphere, which was not evaluated in this study. In other words, mycorrhizal colonization may have enhanced the endophytic colonization of the bacteria or may have facilitated bacterial entrance. Endophytic bacteria may contribute to the control of sedentary plant parasitic nematodes (Hallman *et al.*, 1997). A strain of *C. turbata* has been reported to be endophytic with biological control capability against Fusarium wilt (Musson *et al.*, 1995; Chen *et al.*, 1995).

The present study clearly demonstrates a synergism influence of dual inoculation treatments against *M. incognita* under the described experimental conditions. It is accepted that more complex interactions should provide more stable control achieved with micro-organisms, and the binary association of bacteria and mycorrhizal fungi could be beneficial to plant health and growth. However, the proper selection of both bacterium and AM fungus is very important for a positive effect on plant performance. To better utilize the beneficial effect of the mycorrhizal symbiosis screenings for compatibility with different bacterial strains remain a major task (Alabouvette *et al.*, 2001).

## 6 General discussion

Estimated crop losses due to plant-parasitic nematodes exceed annually $100 billion. The recent de-registration of several chemical nematicides such as DBCP (dibromochloropropane) and EDB (ethylene dibromide) and the phase out of methyl bromide the have led to increasing interest in the use of biological control agents to control those pests (Oka *et al.*, 2000 Chitwood, 2003). Natural enemies of nematodes in the soil are many, including bacteria, fungi, and protozoa. Other types of biological control agents are the rhizospheric and endophytic fungi and bacteria, which may protect plants directly or indirectly rather than through direct parasitism of the nematodes.

Introduction of natural enemies into soil is one possible biological control strategy for plant parasitic nematodes (Sikora, 1992; Kerry, 1993; Kerry, 2000; Oka *et al*., 2000; Meyer and Roberts, 2002; Meyer, 2003). The outcome, however, depends on the interactions of the organisms within particular plant pathogen ecosystems (Roberts and Lohrke, 2003). The more that is known about the behavior of the nematodes in a particular biocontrol interaction system, the easier it will be to establish strategies that optimize biocontrol performance for that particular interaction.

In this work the AMF-RKN interaction in tomato was studied. Several AMF isolates were screened as possible biocontrol agents against the root-knot nematode *M. incognita*.
For an effective reduction of damage caused by nematodes it is necessary that AMF influence the pre- and/or post-infectional nematode/host relationship or that they enhance plant tolerance (Thomson Cason *et al*., 1983).

Suppression of nematode infection by AMF is isolate specific and was not attributed to difference in mycorrhizal colonization. Raising AMF initial inoculation levels enhanced mycorrhization, however, this was not translated into higher degree of nematode suppression. This clearly proves that the ability of AMF to prevent gall formation is not related to their ability to colonize roots and agree with earlier observations (Saleh and Sikora, 1984; Diedhiou *et al.,* 2003). On the hand, the size of nematode galls induced by *M.*

*incognita* was not isolate specific and was sensitive to frequency of AMF colonization. Reduced gall sizes on mycorrhizal plants are attributed to either younger age of the galls, slower development of the female inside the gall, lower degree of hypertrophic development of the gall tissue and/or less nematodes in the tissue. The exact mechanism causing the reduction in gall sizes in mycorrhizal plants is uncertain and requires a close synchronization of juveniles' penetration and estimation of female's size.

The mycorrhizal colonization seems to exert different influences on the different aspects in the nematode life cycle. The first possible recognition event in the AM-nematode interaction (Hatching) was not influenced by mycorrhizal colonization. Root diffusates are not required for substantial hatch of most *Meloidogyne* spp. (Gaur *et al.*, 2000; Zhao *et al.*, 2000). Hatching is more dependent on environmental factors rather than plant signals. In contrast, juveniles were less attracted to (or even repelled by) the mycorrhizal plants. Influence of mycorrhizal colonization on root exudation in relation to chemotaxis of plant pathogens have been reported (Marschner, 1997; Harrier and Watson, 2004). Kairomones attracting nematodes are likely to be of a volatile or water diffusible nature (Perry, 1997; Rühm *et al.*, 2003) and reported to be genus specific and to influence specific developmental stages in the nematode life cycle (Davis *et al.*, 1992). The degree of nematode suppression by the different mycorrhizal isolates was not related to the degree of attractiveness of the roots. An isolate of *G. etunicatum* (36) -that is inefficient in suppressing nematode infection- was as repellent to nematodes juveniles as the efficient AMF isolate (*G. intraradices* 510). Fractionation and comparison of root exudates from mycorrhizal and non-mycorrhizal plants would help to determine whether reduced attractiveness of the mycorrhizal roots is due to altered levels of exudates or to the generation of novel chemicals associated with the mycorrhization and capable to impair the orientation of the juveniles.

Moreover, mycorrhization did not limit the potential infection sites for nematode juveniles, however, delayed the penetration process. Entering the root involves a combination of mechanical piercing by the stylet and enzymatic softening (Hussey, 1985; Wyss, 1992; Francl, 1993, Huang *et al.*, 2003). Lignification of the cell walls due to mycorrhizal

infection is known (Gnavi *et al.*, 1996; Slezack *et al.*, 1999). The delayed penetration can thus be attributed to the lignification of the AM roots. Correlating this observation to the results obtained from attraction assay would suggest that juveniles' orientation could have been affected. Delayed penetration due to juveniles' disorientation suggests that mycorrhization exert influence on both long distance attraction and attraction to the target tissue.

To understand the mechanism involved in AMF suppression of root-knot nematode, AMF performance was assessed in a split root trial. Compared with the controls, application of AMF to one-half of the root system lowered the infection of root-knot nematode in non-mycorrhizal nematode-treated sections indicating enhanced defence in the non-mycorrhizal half.

Proline was considered as a physiological indicator for the AMF-RKN interaction as it is known to accumulate in nematode-induced galls at high concentrations (Lewis and McClure, 1975; Bird and McClure, 1976) and reported to be one of the most influenced amino acids by nematode infection (Sharma and Trivedi, 1996; Mohanty *et al.*, 1999). Proline concentration was positively correlated with the nematode's infection. Considering the reduced nematode infection in mycorrhizal plants and that induced resistance is involved in nematode suppression by AMF would explain the negative influence of AMF on proline accumulation. Induced resistance is reported to be inactivated in tomato by treatment with L-proline (Oka and Cohen, 2001).

The main results of the co-inoculation of tomato with the AM fungus *G. intraradices* 510 and either rhizobacterium SR1or SR6 suggest a synergistic effect of the co-inoculation treatment against *M. incognita*. It is suggested the some rhizobacteria cause a short disruption of nematode infection during early roots penetration (Sikora and Hoffman-Hergarten, 1993). The better performance of the co-inoculation treatment in suppressing nematode infection suggests a combined effect due to mycorrhizal and bacterial inoculation. Thus lack of influence of the singly inoculated bacteria treatments would suggest that disrupting penetration is not enough to suppress infection.

In general, it is expected that AMF would only provide partial levels of nematode control and likely to not to work as fast as chemical pesticides. The implementation of early mycorrhizal inoculation at nursery level can represent an option that is rarely considered for the management of root-knot nematodes. Further investigation on the repellence of nematode juveniles and the delayed invasion due to mycorrhization may be fruitful for possible practical applications in combination with other biocontrol agents that may influence the juveniles to optimize biocontrol performance.

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