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Activity of fungal and bacterial endophytes for the biological control of the root-knot nematode *Meloidogyne graminicola* in rice under oxic and anoxic soil conditions

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This work is dedicated to my family!

Activity of fungal and bacterial endophytes for the biological control of the root-knot nematode *Meloidogyne graminicola* in rice under oxic and anoxic soil conditions

Two endophytic *Fusarium moniliforme* isolates Fe1 and Fe14, an endophytic bacterium *Bacillus megaterium* Bm and a rhizosphere *Trichoderma* isolate T30 with known antagonistic activity toward the root-knot nematode *Meloidogyne graminicola* were studied for bio-enhancement of rice under glasshouse conditions.

The level of colonization of Fe1 and Fe14 in the rice root under oxic and anoxic soil environments was investigated. The fungi were inoculated twice to the rice seeds using seed treatment and soil drenching methods at a rate of 10^6 cfu/ seed and 10^5 cfu/ seedling respectively. Both Fe1 and Fe14 isolates colonized well in the rice roots under oxic and anoxic soil water regimes with colonization rate ranged between 50-89%. The fungi colonized all parts of the roots though the preferable zone was the root periphery. The level of colonization decreased over time, from 56% after 8 weeks to 27% after 12 weeks of incubation. Both isolates did not show consistent effect on the growth of rice.

The mechanisms of action of the endophytic *F. moniliforme* isolate Fe14 was studied intensively under glasshouse conditions. In these experiments, Fe14 was also inoculated twice by seed coating and soil drenching techniques. The fungus reduced nematode penetration into the rice root significantly by up to 55% compared to the control. In a split-root experimental design, the fungus showed induced systemic resistance in rice when one half of the root system was treated with fungal spores while the other half was inoculated with the root-knot nematode. Root exudates from fungal treated plants showed repellent effect toward *M. graminicola* in a plastic test chamber. Fe14 also altered nematode development expressing by significantly higher number of males in fungal treated plants. Furthermore, Fe14 reduced the number of females and number of eggs per female compared to those of the control treatment. In addition, Fe14 exhibited high level of biocontrol under anoxic soil conditions by reducing the total number of nematodes in the endorhiza significantly by 45%.

Influence of inoculation time and method on biocontrol efficacy of Fe14 was also evaluated. In the first test, the ability of Fe14 for early protection of *M. graminicola* was tested in comparison to other antagonistic fungi. Out of the five fungi tested, *F. moniliforme* Fe1 and Fe14, *F. oxysporum* Fo162, *Fusarium* F28 and *Trichoderma* T30, only *Trichoderma* T30 was able to reduce nematode infestation in rice seedlings when both nematode and fungi were inoculated at sowing. However, Fe14 remained its biocontrol activity against the rice root-knot nematode 10 weeks after fungal inoculation. The effectiveness of different inoculation methods of Fe14 was also investigated. Both seed treatment and soil drenching methods led to similarly significant reductions in nematode damage. Double inoculations of Fe14, one at sowing and the other one repeated three weeks later did not result in significantly higher biocontrol level compared to single inoculation at sowing.

To enhance biocontrol efficacy, Fe14 was combined with *Trichoderma* T30 and the endophytic bacterium *B. megaterium* Bm in various greenhouse experiments. The three antagonists were first tested for their compatibility *in vitro*. No clear mutual exclusive was observed in any pair tests. Dual application of Fe14 and T30 *in vivo* reduced nematode infestation significantly compared to the control but the difference between single and combined treatments was not significant. Similarly, when Fe14 was combined simultaneously or in a staggered time manner with T30 and Bm, galling severity caused by *M. graminicola* significantly reduced by 20-70% compared to the control. However, none of the combinations led to significantly higher level of biocontrol compared to single applications and thus, single treatments of each biocontrol agent was adequate.

Wirksamkeit pilzlicher und bakterieller Endophyten für die Bekämpfung der Wuzelgallennematode *Meloidogyne graminicola* an Reis unter aeroben und anaeroben Bedingungen

Für die biologische Kontrolle von *Meloidogyne graminicola* unter kontollierten Bedingungen wurden zwei endophytische Isolate von *Fusarium moniliforme* (Fe1 und Fe14), ein endophytisches Bakterium *Bacillus megaterium* Bm und ein Rhizosphärenisolat *Trichoderma* T30 mit bekannten antagonistischen Wirkungen genutzt.

Die Kolonisationsraten von Fe1 und Fe14 in der Reiswurzel unter aeroben und anaeroben Bedingungen wurden untersucht. Der Pilz wurde zweimal an die Reissamen inokuliert, jeweils durch Samenbeizung und Tauchinokulation mit einer Rate von 10^6 cfu/ Samen und 10^5 cfu/ Pflanze. Beide Isolate Fe1 und Fe14 kolonisierten die Reiswurzeln undter anaeroben und aeroben Bedingungen mit Raten von 50 bis 89%. Der Pilz kolonisierte alle Teile der Wurzel, wobei die hauptsächliche Besiedlung an der Wurzelperipherie lag. Die Kolonisation ging über die Zeit zurück, von 56% nach 8 Wochen auf 27% nach 12 Wochen Inkubationszeit. Beide Isolate zeigten keinen Effekt auf das Wachstum der Reispflanzen.

Die Wirkungsweise des Endophyten *F. moniliforme* Isolat Fe14 wurde unter Gewächshausbedingungen intensiv untersucht. In diesen Experimenten wurde der Pilz ebenfalls zweimal durch Samenbeizung und Tauchinokulation zu den Pflanzen gegeben. Der Pilz reduzierte die Nematodenpenetration signifikant um bis zu 55% im Vergleich zur Kontrolle. Durch ein experimentelles Design in welchem die Wurzeln räumlich voneinander getrennt wurden, wurde eine induzierte Resistenz an Reis nachgewiesen. Hierbei wurde nur eine Hälfte des Wurzelsystems mit Sporen des Endophyten behandelt und die andere Hälfte mit Nematoden inokuliert. Wurzelexudate der pilzlich behandelten Pflanzen zeigten eine abweisende Wirkung gegen *M. graminicola* in Plastiktestkammerversuchen. Fe14 verursachte eine Verschiebung des Geschlechtsverhältnisses. Die Anzahl der Weibchen und die Anzahl der Eier pro Weibchen wurde im Vergleich zur Kontrollvariante reduziert. Zusätzlich wurde eine sehr starke biologische Konrolle durch Fe14 unter anaeroben Bedingungen erziehlt. Die Anzahl der Nematoden in der Endorhiza wurde um 45% reduziert.

Der Einfluß der Inokulationszeit und -methode auf biologische Kontrollaktivität von Fe14 wurde ebenfalls untersucht. Im ersten Test wurde die Fähigkeit von Fe14 für die frühzeitige Kontrolle von *M. graminicola* im Vergleich zu anderen antagonistischen Pilzen untersucht. Von den fünf getesteten Pilzen, *F. moniliforme* Fe1 und Fe14, *F. oxysporum* Fo162, *Fusarium* F28 und *Trichoderma* T30, konnte nur *Trichoderma* T30 die Nematodenpopulation reduzieren, wenn Nematode und Pilz zur Saat inokuliert wurden. Die Effektivität verschiedener Inokulationsmethoden wurde an Fe14 ebenso untersucht. Sowohl die Samenbeizung als auch die Tauchinokulation führten zur signifikanten Reduktion der Nematodenpopulation.

Um die biologische Kontrollaktivität zu erhöhen, wurde Fe14 mit *Trichoderma* T30 und *B. megaterium* kombiniert. Dadurch wurde die Vergallung der Wurzeln um 20-70% signifikant reduziert, jedoch zeigten sich keine Unterschiede in der Reduktion der Nematodenpopulation durch einzel oder kombinierte Inokulation der verschiedenen Organismen.

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CHAPTER 1: General introduction

1. The rice crop

1.1 General information

Rice is the most important cereal crop worldwide since it provides staple food for more than half of the world's population (FAO, 2009). Of the 25 species distributed in parts of Asia, Africa, Australia, Central and South America, only *Oryza sativa* L. and *O. glaberrima* Steud are cultivated extensively. The Asian rice, *O. sativa*, is grown worldwide and was believed to have been domesticated in the northeast and southeast regions of the continent around 5000 years ago. Asia now accounts for more than 90% (622 million tons) of world rice production with China, India and Indonesia producing more than half of the total volume (FAOSTAT, 2008).

The genus *Oryza* belongs to the tribe Oryzeae of the family Poaceae (Gramineae). The species *O. sativa* consists of numerous ecotypes and several genetic groups. The ecotypes are divided into the *indica*, *japonica* and *javanica* types based on morphological and physiological criteria. The traditional varieties of *indica*, most widely distributed in Africa, are grown as a rainfed crop and on submerged land in the tropics. The *japonica* ecotype includes the varieties growing in tropical upland regions and temperate zones. The *javanica* ecotype is well adapted to tropical, rainfed cultivation and to subtropical, submerged cropping (Schalbroeck, 2001)

1.2 The rice plant

O. sativa (2n = 24) is an annual grass with erect stems and a terminal panicle bearing hermaphroditic flowers. Mature plants consist of a root system, stem, 3-10 productive tillers bearing panicles and about 10-20 leaves.

The roots are massed in the first 20 - 25 cm of soil. Root depth may be as little as 15 cm in heavy soils and can reach more than 50 cm in light soils. The presence of large, intercellular spaces in the cortical parenchyma of the roots enables their oxygenation and gives them the ability to grow under flooded conditions.

The growth cycle of rice can be divided into three phases: vegetative, reproductive and ripening. The vegetative phase stretches from germination to the end of tillering. The reproductive phase covers panicle initiation, rise of the panicle up the stem (booting), emergence of the panicle (heading), flowering and fertilization. The ripening (maturation) phase starts after fertilization, continues through grain filling, and terminates at harvest time. The varieties are usually classified according to the length of the growth cycle into early or short-cycle rice (90 to 120 days), medium-cycle rice (120 to 150 days) and late or long-cycle rice (more than 150 days). The differences in growth duration are determined by changes in the length of the vegetative phase. For example, IR64 which matures in 110 days has a 45-day vegetative phase, whereas IR8 which matures in 130 days has a 65-day vegetative phase (IRRI knowledge bank a)

1.3 Rice cropping systems and cultivation techniques

By taking the water supply as the point of reference, five main types of cultivation can be distinguished: upland, low land rain-fed, irrigated, deep water and tidal wetlands rice (CORIFA, FAO).

Upland rice cultivation implies that the water is supplied by rainfall or ground water. Upland rice is grown on the plains as well as on variably sloping land at all altitudes. This cropping system covers only 9% of the rice area in Asia whereas in Africa it accounts for 60% of the rice area (Schalbroeck, 2001).

In lowland rain-fed rice, water supply to the rice plants is intermittently provided by rainfall, runoff or underground water. The rain-fed lowland rice fields are usually bunded. The bunds serve to retain floodwaters, as well as rainwater which falls during the growing season. Rain-fed lowland rice may suffer at times from both drought and flooding.

Irrigated rice plants are constantly supplied with full water levels throughout the growing season. Water in the rice fields is controlled by bunds, with a system of irrigation canals and drains. The water may be supplied via streams, rivers, or underground water from wells. In many irrigated rice areas, rainfall supplements

irrigation water. This is the most widespread system in Asia where 93% of the area under rice is irrigated.

Deepwater and floating rice are grown in the low lying lands of the deltas of large rivers such as the Mekong in Vietnam, Cambodia and Thailand, the Ganges-Brahamaputra-Megna in Bangladesh and along the Niger River in West Africa. The varieties adapted to the deeply flooded areas are sometimes referred to as floating rice. These varieties are characterized by their ability to grow under water inundation to a depth of 1 - 4 m and are therefore fast-growing varieties (up to 20 cm/day). The plant elongates with increasing water depth, but retains a rooted foot hold in the soil. Floating rice varieties also form adventitious roots from the nodes which are able to absorb nutrients directly from the floodwater (Schalbroeck, 2001). Occurring over a small area is the tidal wetland ecosystem which is located near sea coasts and inland estuaries. This rice system is directly or indirectly influenced by tides.

Crop establishment practices in rice vary from direct sowing of dry, wet, or shallow-flooded soils to the establishment of seedlings in a seedbed or nursery followed by transplanting. Direct sowing is a common practice in upland and lowland rice production when water is in short supply at the start of cultivation. In upland rice cultivation, sowing is timed to let the plants develop strong roots before a possible dry period and to make sure that flowering takes place in the rainy season and maturity coincides with the following dry season. The seed rate ranges from 30 - 120 kg/ha with the average of 60-80 kg/ha.

In irrigated rice cultivation, the sowing date is less dependent on rainfall. If the rice field is dry at the start of cultivation, it is sown with dry seeds. Conversely, if the rice field is under water (a practice which allows for early weed control), direct sowing must be carried out with germinated seeds because the seeds need a high oxygen environment during germination. In this case, the technique can be called wet seeding. Germinated seeds for wet seeding are broadcast at the rate of 100 - 200 kg of seeds/ha in 2 - 5 cm of water. The water level is kept at 3-5 cm until the plants are 15 - 20 cm tall to encourage tillering. The water level is then raised to a height of 10 - 20 cm.

Raising plants in a nursery or seed bed and then transplanting them is the most common method of establishing an irrigated rice crop. The surface area of the nursery and that of the rice field are roughly in the proportion of 1 to 25; 30 - 60 kg of seed are needed per ha of transplanted rice, according to the varieties used and the chosen spacing. There are several kinds of rice nurseries such as "Modified Mat Nursery" or "Reduced Area Wet bed Nurseries". The choice of nursery type depends on the area, space, quality of seeds and other techniques and equipment. Transplanting can be done mechanically or manually. Rice seedlings grown in a nursery are pulled and transplanted into puddled and leveled fields 15-40 days after seeding (IRRI knowledge bank a).

2. Nematode parasites

2.1 Diversity of plant parasitic nematodes

Many species of nematodes are associated with rice but only a few are considered as economically important pests (Bridge et al., 2005). The plant parasitic nematodes of rice can be divided into two groups according to the plant parts infected: the stem, leaf and root nematodes. One of the foliar parasites *Ditylenchus angustus*, or the "Ufra nematode", occurs mainly in river deltas on both deepwater and lowland rice in Bangladesh, Myanmar, Vietnam, India and Malaysia. The nematode causes yellowish or whitish splash patterns on the invaded areas of leaf sheaths, retards panicle formation and spikelet filling processes and consequently causes yield loss up to 30% per field in the North-eastern states, Assam and West Bengal of India (Prasad et al., 1987). White tip disease caused by *Aphelenchoides besseyi* Christie, was recorded in rice producing regions of Asia and Africa. Infected plant mature late and have sterile white panicles. Yield loss in infected fields varies from 4.9% in USA by up to 50% in China (Bridge et al., 2005).

Important root parasites include species of *Meloidogyne* and *Hirschmaniella*. *Hirschmaniella* species, known as rice root nematodes occur in the majority of rice growing regions. They are migratory endoparasites of roots. Unspecific above ground symptoms make it difficult to diagnose the causal agent instantly and thus the level of actual damage may be underestimated, and is often incorrectly attributed to poor soil

fertility or other abiotic stress. Roots invaded by *Hirschmaniella* spp. turn yellowish brown and rot. It has been estimated that *Hirschmaniella* can cause up to 25% of yield loss in an infected field (Bridge et al., 2005). All nematodes belong to the genus *Meloidogyne* cause swellings and galls in the root system. The yield loss depends on the level of infection, which is largely a function of the amount of time the rice root grows under non-flooded conditions. The root-knot nematode *Meloidogyne graminicola*, one of the most important sedentary nematode in rice, will be discussed more detailed in the next section.

2.2 The rice root-knot nematode *Meloidogyne graminicola*

The rice root-knot nematode belongs to the family *Heteroderidae* and is one of the most economically important nematodes affecting rice. It has been reported to cause significant yield losses of 20-50% in many regions of rice production: India, Bangladesh, Philippines, Thailand, Vietnam, Cambodia and Indonesia (Manser, 1968; Prasad et al., 1987; Arayarungsarit, 1987; Netscher and Erlan, 1993; Prot et al., 1994; Cuc and Prot, 1992; Soriano and Reversat, 2003; Padgham et al., 2004). *M. graminicola*, like other root-knot nematodes causes swellings and galls in the root systems. Infected rice root tips show swollen and hooked like symptoms. The nematode can retard plant growth, cause unfilled spikelets, reduce tiller development and cause chlorosis and wilting symptoms under upland and intermittently flooded conditions.

The life cycle of *M. graminicola* varies considerably in different environments, ranging from a very short life cycle of 19 days at temperatures ranging from 22-29^{o}C in Bangladesh (Bridge and Page, 1982) to up to 51 days in some regions in India (Rao and Israel, 1973). The nematode experiences 4 molts throughout its life cycle. The first molt takes place inside the egg and newly hatched juveniles accumulate round the roots in the zone of elongation. Most juveniles also hatch inside the gall and re-infect the same root by moving to a new feeding site (Mulk, 1976). Females of *M. graminicola* remain within the galled roots and eggs are deposited in the cortex inside the egg masses. Up to 50 females can be found in a single gall, indicating that the level of infestation can be very high (Bridge et al., 2005).

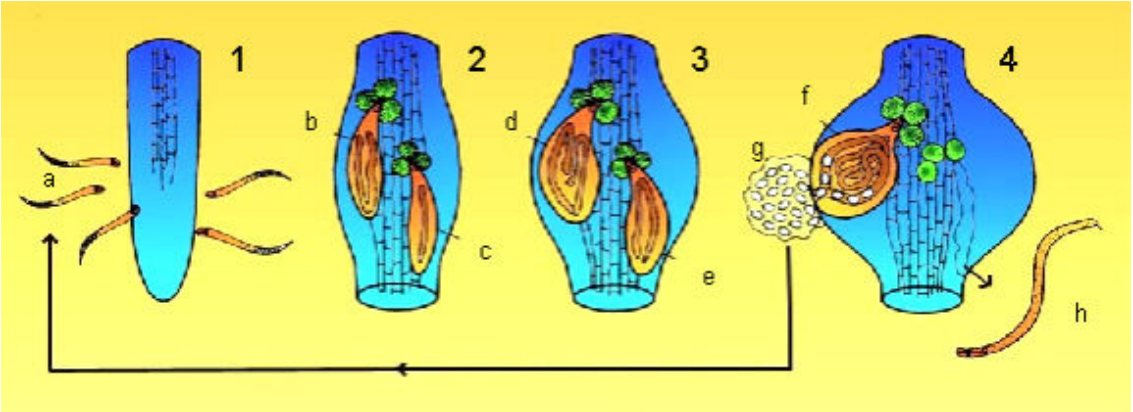

Figure 1.1: Life cycle of the rice root-knot nematode *Meloidogyne graminicola.*

(a) Second stage juveniles penetrate the roots closely behind the root tip and migrate to the vascular cylinder; immature female (b) and a male (c) of the J3 larval stage; females (d) and males (e) in the J4 stage; the male (h) changes its shape in the last molt and leaves the root; (g) the female lays its eggs in a gelatinous matrix (IRRI knowledgebank b).

The second stage juvenile of *M. graminicola* is the infective stage. The juveniles enter the rice roots behind the root tips and start feeding when they reach the cortex where they swell and become sedentary. Root-knot nematodes induce changes in the cells around their head to increase their nutritional value. Cells fed on by juveniles enlarge and their nuclei repeatedly divide to form multinucleate "giant cells". The nuclei within giant cells become polyploidy, further increasing the metabolic capacity of the feeding site. These cells provide a constant supply of nutrients to the nematode (Trudgill, 1997).

The sex of juveniles is not predetermined. Those developing under limited nutrient supply conditions and poorly developed giant cells or faced with a resistant variety become males whereas those with normal giant cells become females. This adaptation is thought to prevent the population from increasing to large self-limiting densities, thereby preventing the host from being killed (Trudgill, 1997). This mechanism is also used to increase genetic diversity and to help form races able to overcome resistant varieties. Study on the effect of inoculations with single juveniles on release of progeny of *M. graminicola* confirmed that this species is able to reproduce by parthenogenesis.

71-73% of the seedlings released second stage juvenile progeny after 84 days inoculation with single juvenile (Reversat and Fernandez, 2004).

Juveniles survive at temperatures of 20-26^{o}C for up to 5 months in bare soil (Bridge and Page, 1982). *M. graminicola* was found to survive longer at 20^{o}C compared to 26^{o}C (Soomro, 1994). The survival rate is also affected by moisture, osmotic pressure, pH and other environmental factors.

M. graminicola, like many other species of *Meloidogyne* has a wide host range. However this nematode mostly affects gramineous species like rice, wheat, sorghum and grasses. The nematode is also frequently reported to be an important pest in rice-wheat cropping systems in South Asia such as Nepal, Bangladesh, Pakistan and India (Sharma, 2001; Bridge at al., 2005; Pokharel et al., 2006). The root-knot nematode is a possible causal candidate contributing to the observed yield decline in Nepal rice-wheat cropping systems. However, proper management is often neglected due to a lack of conspicuous above ground symptoms (Bridge et al., 2005; Pokharel et al., 2006). Other agricultural crops such as peanut, onion or potato can be alternative hosts for this nematode. *M. graminicola* can cause serious damage to rice seedlings and consequently cause significant yield loss in upland and lowland rain-fed rice. Moreover, the nematode possesses the capacity to infect, survive, and re-infect the rice root as soils fluctuate between oxic and anoxic states (Gaur et al., 1996; Sharma, 2001; Bridge et al., 2005). Initial infection occurs at planting. Flooding then prevents the nematode from further entering the rice plants. However, whenever water recedes, *M. graminicola* again reactivates and infects plants and can cause devastating damage (Bridge and Page, 1982; Padgham et al., 2003). The nematode can develop, reproduce and complete many life cycles within the rice roots for the entire crop period once established, regardless of the soil oxygen level. It has been reported that the density of second stage juveniles of *M. graminicola* is 2 to 10 times higher in rice growing under anoxic conditions than that of rice growing in oxic conditions (Tandingan et al., 1996; Soriano et al., 2000).

2.3 Current control status of the rice root-knot nematode

Different management strategies have been used to control plant parasitic nematodes with various degrees of success. The use of chemical nematicides, either fumigants or nonfumigants is an effective and simple approach. However, most chemical nematicides are highly toxic to humans and animals and have negative effects on the environment when misused (Sikora and Fernandez, 2005). In addition, the high cost of chemical control restricts the use of nematicides in low input crops like rice or it is only applicable on a small scale such as seedbed or nursery treatment (Prasad and Rao, 1976a, 1976b). Cultural practices used to control the rice root-knot nematode include crop rotation, fallowing, flooding or incorporation of organic amendments (Rahman, 1990; Rahman and Miah, 1993; Prot et al., 1994; Prot and Matias, 1995; Debanand et al., 1999). Other control measure such as using neem can be effective but application of mulches is quite complex and their use is limited to specific region. These control options are usually not cost effective and are not applicable in many regions where: 1) rice is a mono crop, 2) organic matter is not available, 3) the method is expensive or 4) long term flooding of the soil is not possible. The wide host range of *M. graminicola* also limits crop rotation in many situations. Resistant lines of rice against root-knot nematodes have been reported (Soriano et al., 1999). However, no resistant commercial varieties are available on the market. Therefore, the development of an effective and cost saving alternative control option against the rice root-knot nematode is highly desired.

3. Biological control of plant parasitic nematodes

Biological control of plant parasitic nematodes has been defined as a reduction in nematode population density which is accomplished through the action of living organisms other than nematode resistance to host plants. It occurs naturally, through the manipulation of the environment or following the introduction of antagonists (Sikora, 1992). Biological control is a promising alternative to expensive and toxic nematicides, limited and inadequate cultural control practices and the lack of resistant varieties. In order to apply biocontrol technology successfully, the following issues must be

considered: 1) which organism is the most effective under local conditions, 2) which crop is suitable for biological control, 3) which is the targeted nematode species and 4) how to apply the biocontrol agents in IPM systems to optimize control levels (Neuenschwander et al., 2003).

Biological control of plant parasitic nematodes using their natural enemies has been studied extensively in the last two decades and many successful cases have been reported but few are used in the field. The use of arbuscular mycorhizal fungi (AMF), rhizobacteria, endophytic bacteria, rhizosphere and endophytic fungi as biological control agents has been well documented on many food, vegetable and cash crops (Hallmann and Sikora, 1994a; Schuster et al, 1995; Niere et al., 1999; Pocasangre, 2000; Meyer et al., 2000; Khan et al., 2001; Sharon et al., 2001; Masadeh et al., 2004; Reimann, 2005; Vu et al., 2005; Rumbos et al., 2006; zum Felde et al., 2006; Dababat and Sikora, 2007; Mendoza, 2008; Chaves et al., 2009; Elsen et al., 2009; Le et al., 2009). The application of biological control agents for the control of plant parasitic nematodes is often targeted at the planting material such as in seed treatment, seed bed incorporation, seedlings and banana suckers drenching or at transplanting time to increase effectiveness and reduce the cost of treatment (Sikora, 1992; Sikora et al., 2007).

The soil is a nourishing environment for a vast number of micro fauna and flora. Natural soil ecosystems contain a certain spectrum of biodiversity which is considered important in protecting a plant from disease and nematode attack. Bacteria and fungi are among the most dominant soil-borne groups and some of them have shown great potential as biological control agents for plant parasitic nematodes. Natural antagonists interfere with the nematode's ability to find, penetrate and complete its life cycle in the host, through direct competition, antibiotics as well as through the induction of systemic resistance (Stirling, 1991; Sikora, 1992; Kerry, 2000; Sikora et al., 2007).

Many bacterial species have been evaluated for their antagonistic activity against a wide range of plant parasitic nematodes. Bacteria can be isolated from plant tissues, soil, and plant debris or from the nematode body. Well studied bacteria include *Pasteuria*

penetrans, species of *Bacillus* and *Pseudomonas* or *Burkholderia cepacia* (Chen and Dickson, 1998; Qiuhong et al., 2006). Various modes of action of the bacteria toward plant parasitic nematode have been demonstrated: parasitism, interference with nematode-host recognition, competition for nutrients and induced systemic resistance (Hasky-Günther et al., 1998; Siddiqui and Mahmood, 1999; Hallmann, 2001; Sikora et al., 2007). In many cases, antibiotics or the toxic secondary metabolites produced during fermentation processes show nematicidal activity. In addition, some bacterial strains such as *B. firmus*, *P. penetrans* and *Burkholderia cepacia* are available on the market as biocontrol agents (Meyer and Roberts, 2002).

Several fungal species are known to be egg pathogens of plant parasitic nematodes. More than 150 fungal species have been isolated from cysts, females or eggs of nematodes but only a small fraction have been tested (Kerry, 1988). Among those, *Paecilomyces*, *Trichoderma*, *Podochia* (syn. *Verticillium*) and *Fusarium* are the most well studied genera (Jatala, 1986; Kerry, 2000; Rumbos et al., 2005; Sikora et al., 2007; Kiewnick, 2009). The egg pathogens *Paecilomyces lilacinus* and *P. marquandii* have proven antagonistic activity towards eggs and give good nematode control in several crops such as tomato and banana. For example, the isolate *Paecilomyces lilacinus* 251 showed high biocontrol level against *M. incognita* in tomato (Rumbos et al., 2006) and against *R. similis* in banana (Mendoza and Sikora, 2009). Furthermore, the genus *Trichoderma* is worldwide in occurrence and is easily isolated from soil and organic matter. Some *Trichoderma* species such as *T. virens*, *T. viride, T. harzianum* have been used to successfully control the root-knot nematode on vegetable crops such as tomato or bell pepper (Windham et al., 1989; Spiegel and Chet, 1998; Meyer et al., 2000; Sharon et al., 2001). In addition, *Trichoderma* species are also frequently reported for their growth promoting effect on the host plants. Other fungi such as *Cylindrocarpon destructans* (Crump, 1987) and *Dactylella oviparasitica* (Olatinwo et al., 2006) have been also demonstrated to be antagonistic fungi of nematodes.

Arbuscular mycorhizal fungi (AMF) are obligate symbionts which colonize the roots of about 80% of vascular plants. The AMF enhance growth and survival of many plant species through improvement of water and nutrient uptake by the hosts. Moreover,

AMF can also reduce the occurrence and effect of soil pathogens. Therefore, numerous studies on the potential of AMF as biocontrol agents against wide range of plant parasitic nematodes have been carried out (Masadeh et al., 2004; Reimann et al., 2008; Elsen et al., 2009). AMF protect plants from nematode attack through several modes of action such as induced systemic resistance, competition for nutrients and space within the host plants as well as enhancement of plant growth and health.

Research has recently shifted from the above to fungal antagonists that reside endophytically in the host plants (Pocasangre et al., 2001; Sikora et al., 2007, Hallmann et al., 2009). There are four possible forms of activity of endophytic fungi on a specific plant and nematode: 1) having no effect on plant growth and nematode infection, 2) being pathogenic to the plant with no effect on nematodes, 3) being pathogenic to plant and nematodes, 4) promoting plant growth and nematode control activity (Schuster et al., 1995). Several fungal endophytes have been studied for the biocontrol of root nematodes and success has been recorded under greenhouse conditions. For example, a mutualistic strain of *Fusarium oxysporum* (Fo162) which was isolated from field tomato in Kenya has been shown to reduce root-knot nematode gall formation and egg masses on tomato (Sikora, 1992; Hallmann and Sikora, 1994b, 1996; Dababat and Sikora, 2007). Fo162 also significantly reduced the infestation of the burrowing nematode *Radopholus similis* on banana while promoting plant growth (Vu et al., 2006; Mendoza and Sikora, 2009). Other endophytic *F. oxysporum* or *Trichoderma* species isolated from banana roots also showed high level of biocontrol against *R. similis* under field conditions (zum Felde et al., 2006).

However, until now, there have been few investigations on the interaction between endophytes and nematodes on gramineous species. The study on the interaction between the fungal shoot endophyte *Acremonium* species and nematodes on tall fescue showed lower rates of reproduction of several species of plant parasitic nematodes (Kimmons et al., 1990; West et al., 1988). There has also been limited research on the biological control of *M. graminicola* in rice, despite its widespread occurrence in major rice producing areas. These studies have been primarily aimed at controlling the parasite in

the rhizosphere before it penetrates the root (Debanand et al., 1999; Duponnois et al., 1997; Singh et al, 2007).

Recently, Padgham and Sikora (2007), Singh et al. (2007) and Le et al. (2009) have demonstrated that endophytic bacteria and fungi isolated from the rice root can reduce *M. graminicola* infestation in rice. However, the study of endophyte-based control systems that are effective in oxic and anoxic environments is non existent. Therefore, the development of a model biocontrol system that can be applied to the important nematodes of rice like *M. graminicola* and *Hischmaniella* would be of great importance for growers.

4. Scope of the study

The overall goal of the present study was to investigate the activity of isolates of the endophytic fungus *Fusarium moniliforme* (Fe1 and Fe14), the endophytic bacterium *Bacillus megaterium* (Bm) and the rhizosphere fungus *Trichoderma* (T30) for the biological control of the rice root-knot nematode *Meloidogyne graminicola* under oxic and anoxic soil water environments. The specific objectives of the study were to:

1) Investigate the colonization activity of the endophytic isolates of *Fusarium moniliforme* Fe1 and Fe14 in rice roots under oxic and anoxic environments.

2) Determine the effect of the endophytic fungi Fe1 and Fe14 on rice growth

3) Ilucidate the mechanisms of action of Fe14 toward *M. graminicola* under oxic soil conditions.

4) Study the influence of inoculation time and method of application on biocontrol efficacy of Fe14

5) Evaluate the biocontrol activity of combined applications of Fe14 with the endophytic bacterium *Bacillus megaterium* Bm, and the rhizosphere fungus *Trichoderma* T30

CHAPTER 2: General materials and methods

General materials and methods are described in this chapter whereas specific techniques and procedures employed in individual experiments are described within the respective chapters.

1. Biological control agents

1.1 Fungal isolates

1.1.1 Origin

Five fungal strains, mainly isolated from Vietnam soils were used in various tests against *M. graminicola*

Table 2.1: The biological control agents used in this study

Code	Isolates	Host plants	Origin
Fe1	*Fusarium moniliforme*	Rice root	Vietnam
Fe14	*Fusarium moniliforme*	Rice root	Vietnam
F28	*Fusarium moniliforme*	Rice rhizosphere	Vietnam
T30	*Trichoderma*	Rice rhizosphere	Vietnam
Fo162	*Fusarium oxysporum* 162	Tomato root	Kenya

The endophytic fungi Fe1 and Fe14 were isolated from the cortical tissue of surface disinfected rice roots. F28 and T30 were isolated from the rhizosphere of rice roots grown in Vietnamese soil samples (Le et al., 2009). The antagonistic isolate *Fusarium oxysporum* Fo162 was isolated from the root cortex of a tomato plant growing in Kenyan soil.

1.1.2 Culturing and storage of the fungi

All of the fungi were stored at -80^{o}C in Cryobank storage vials (CRYOBANK ^{TM}, MASTE Group Ltd., Merseyside, UK). Inoculum of each fungus for experimental purposes was prepared by transferring a frozen Cryobank bead stored at -80^{o}C and

streaking it over a Petri dish containing 100% PDA (Potato Dextrose Agar) supplemented with 150 ppm of the antibiotics Streptomycin sulphate and Chloramphenicol to suppress bacterial contamination. The fungal cultures were incubated at 25^{o}C in darkness for 3-4 weeks for production of spores. On the test day, 10 ml of tap water was added to the culture plate and the mycelia and conidia were scraped from the mycelia surface with a Drigalski spatula. The suspension was passed through a four-layer cheese cloth to separate fungal spores from mycelia. The spore concentration was determined using a Fuchs-Rosental hemacytometer and then adjusted to the desired density with tap water.

1.2 Bacterial isolate

1.2.1 Origin

The bacterium *Bacillus megaterium* isolate Ni5SO11 (Bm) used in this research originated from a rice plant growing in soil of a rice producing region in Taiwan (Padgham and Sikora, 2007).

The bacterial inoculum was stored in glycerol solution at -20^{o}C. For short term use, the bacterium was stored at 4^{o}C on 100% TSA.

1.2.2 Culturing

The *B. megaterium* inoculum was produced by first pre-culturing the bacteria on 100% TSA at 28^{o}C for 24 hours. A loop full of bacteria was then transferred to a sterilized liquid culture of TSB which was placed on a rotary shaker (120 rpm, 28^{o}C) 1 day prior to inoculation. The bacterial culture was then centrifuged at 5000 rpm for 20 minutes and the bacterial pellet was re-suspended in quarter-strength Ringer's solution. Bacterial density was adjusted to a double optic density at 560 nm, and the bacterial suspension was then diluted 10 times with Ringer's solution. The final concentration of *B. megaterium* was approximately 10^{7} cfu/ ml, as determined by dilution plating.

1.2.3 Determination of colonial forming unit (cfu)

The actual concentrations of the bacterial and fungal suspensions were determined using a spiral plater (Eddy-Jet, IUL Instruments, Germany). The bacterial solution after adjusted with ¼ Ringer solution was diluted 10, 100 and 1000 times. Then, the original and diluted bacterial solutions were placed in the spiral plater and a subsample of 37.3 µl was automatically plated on 50 % Tryptic Soy Agar (TSA) for bacteria or 100% PDB for fungi. The number of colonies was counted everyday for a period of 14 days. CFU was then determined using a counter mat supplied by the manufacturer.

2. Nematode

2.1 Origin and culture of *M. graminicola*

A population of *M. graminicola* isolated from upland rice in Bangladesh was supplied by Dr. John Bridge, CABI, United Kingdom. It was maintained on the susceptible rice variety BR11 growing in autoclaved soil under glasshouse conditions at 28^{o}C ± 5, 12-hr light period in the Section of Nematology in Soil Ecosystems, Phytomedicine, INRES, University of Bonn. Five to six week-old seedlings grown in sterilized sandy soil were inoculated with freshly hatched juveniles (J2) to obtain inoculum for experiments. The infected plants were ready for egg extraction after 8 weeks. Plants were watered daily and fertilized weekly with full strength Yoshida solution.

2.2 Preparation of nematode inoculum

Approximately one week prior to nematode inoculation, nematode infected rice plants were removed from soil of the stock culture and washed with tap water. Root systems were cut into small pieces and macerated for 3 minutes in 1% sodium hypochlorite NaOCl (Hussey and Barker, 1973) in a blender with alternative intervals of 10 seconds macerating and 30 seconds pause to release eggs from egg sacs. The suspension was poured onto a 45 µm mesh sieve placed on top of 25 µm sieve where eggs were collected. The nematode eggs were washed under running tap water for about 9 minutes to remove excessive NaOCl solution. The egg suspension was then gently transferred onto a double layer milk filter placed over a sieve and then submerged into water in a

plastic tray. The tray was kept at 25^{o}C for 7-10 days to allow egg hatching. On the test day, fresh second stage juveniles (J2) were collected for inoculation.

2.3 Determining nematode penetration rate

The roots were washed carefully to remove all the soil particles. Roots were placed in 100 ml plastic vials and then submerged in 1% Fuchsine acid solution (Sikora and Schuster, 2000). Approximate 8 vials containing these root samples were placed in a microwave oven and then heated for about 3 minutes. The stained roots were then kept in the cold room at 4^{o}C overnight to intensify the staining process. To determine the number of nematodes inside the roots, the Fuchsine acid solution was first removed from the vial, and the roots were washed gently under running tap water. Roots were then cut into 1 cm small pieces and macerated for 2 minutes by a commercial blender (Ultra Turrax). The macerated root suspension was diluted up to 100 ml in tap water and a 10 ml subsample was taken to determine the number of nematodes that penetrated using a binocular microscope.

3. Culture media

10% and 100% Potato Dextro Agar media (PDA) were used for fungal isolations and spore production in all experiments. The culture media, unless otherwise specified contained 150 ppm of Streptomycin and Chlorophenicol to prevent bacterial contamination.

<u>100% Potato Dextrose Agar (DIFCO)</u>

24 g	Potato Dextrose Broth
18 g	Agar
1000 ml	Deionized water

<u>10% PDA medium contains</u>

2.4 g	PDB
18 g	Agar
1000 ml	Deionized water

Tryptic Soy Agar (TSA)

15 g	Agar
30 g	Tryptic Soy Broth (TSB)
1000 ml	Deionized water

Tryptic Soy Broth (TSB)

30 g	Tryptic Soy Broth (TSB)
1000 ml	Deionized water

Root stain solution

2 g Fuchsine acid powder + 198 ml water

Lactic acid solution

1750 ml	Lactic acid
126 ml	Glycerine
124 ml	Tap water

1% of the Fuchsine acid added to lactic acid solution

4. Seed coating

Rice seeds were surface sterilized in 75% ethanol for 45 seconds and then in 1.5% NaOCl for 5 minutes followed by several rinses in sterilized tap water. The sterilized seeds were then pre-germinated on wet filter paper placed in 9 cm Petri dishes in the dark at 28^{o}C for 3-5 days. The fungal mycelia and spores were coated onto these 3-day old germinating seeds using a 2% methyl cellulose solution over a 2 hour-period of constant agitation.

To determine the cfu of *Fusarium moniliforme* isolate per seed, a coated seed was first placed in 10 ml of sterilized tap water. This suspension was vortexed well and then diluted to factors of 10, 100 and 1000 times. Colony forming units of all of these suspensions was determined using the spiral plater as described previously.

5. Plant growing conditions

All experiments were conducted under greenhouse conditions at 28^{o}C (± 15), 12-hr light period and with 20-50% humidity. The rice variety BR11, an irrigated rice variety from Bangladesh was used in all experiments.

6. Soil preparation

A mixture of sand and field soil (v/v=2:1) was used for all experiments. The substrate was always autoclaved at 121^{o}C for 60 minutes. The soil was given a period of at least 7 days to allow for the soil to release any toxic gases produced during autoclaving.

7. Fertilizer

A quarter-strength Yoshida solution (Yoshida, 1976) was used to fertilize the seedlings when they developed the third leaf. Half strength Yoshida solution was used from week 3 to week 5. After 5 weeks rice plants were fertilized with full strength Yoshida solution. The pH of the Yoshida solutions was always adjusted to 5.0 using 32% HCl or 3 M KOH solution.

Table 2.1: Composition of fertilizer solution (Yoshida, 1976)

Elements	Reagents	Concentration of element nutrient solution (mg/L)
N	NH_4NO_3	40.00
P	$NaH_2PO_4.2H_2O$	10.00
K	K_2SO_4	40.00
Ca	$CaCl_2$	40.00
Mg	$MgSO_4.7H_2O$	40.00
Mn	$MnCl_2.4H_2O$	0.50
Mo	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.05
B	H3BO3	0.20
Zn	$ZnSO_4.7H_2O$	0.01
Cu	$CuSO_4.5H_2O$	0.01
Fe	$FeCl_3.\ 6H_2O$	2.00

8. Statistical analysis

All data were subjected to analysis of variance using SPSS 11.5 for Windows. Differences among treatments were tested using one way analysis of variance (ANOVA) followed by T-test for mean comparison if the F-value was significant. Mean comparisons were analyzed by Least Significant Difference (LSD) at the 5% level of significance. After verifying homogeneity of variances, the data of repeated experiments were pooled for statistical analysis when appropriate; otherwise data was transformed and analyzed. Graphic presentations were made with Microsoft Excel.

CHAPTER 3: Endophytic colonization and growth promotion in rice

1. Introduction

The genus *Fusarium* is a common soilborne fungus and is widely distributed in cultivated soils around the world. It includes a large diversity of species which can be either pathogenic or non-pathogenic to crop plants (Alabouvette et al., 2001; Olivian et al., 2003). Some species such as *F. oxysporum*, *F. solani* or *F. moniliforme* are important pathogens in many crops such as tomato, rice, maize and other vegetable crops. However, some of them were also demonstrated to live endophytically in the root tissue and display non-pathogenic symptoms on the hosts. These endophytic strains are often considered mutualistic and are an important source for biological control (Backman and Sikora, 2008; Sikora et al., 2007).

One of the best studied endophytes used for biological control against a wide range of plant pathogens is *F. oxysporum*. The fungus resides in healthy plant tissues without causing any damage. The evidence that non-pathogenic endophytic *F. oxysporum* isolates are able to reduce *Fusarium* wilt can be traced back in the early 1970s (Smith and Snyder, 1971; Toussoun, 1975). Since then, many strains of *F. oxysporum* have been studied for their ability to control *Fusarium* wilt disease in many crops worldwide (Biles and Martyne, 1989; Kroon et al., 1991; Minuto et al. 1995; Leeman et al., 1996; Olivian and Alabouvette, 1997, 1999; Fuchs et al., 1999; Alabouvette et al., 2001; Olivian et al., 2003). More recently, endophytic strains of *F. oxysporum* have been reported to successfully control a wide range of plant parasitic nematodes in different crops. Their biological control activity and colonization behavior in these host plants have been studied (Hallmann and Sikora, 1994; Niere et al., 1999, Niere, 2001; Pocasangre, 2000; Vu et al., 2006; zum Felde et al., 2006; Dababat and Sikora, 2007; Mendoza and Sikora, 2009).

In comparison to *F. oxysporum*, *F. moniliforme* Sheldon is also a common soil fungus and is often reported as an economically important pathogen in several crops such as maize and rice. This species is one of the most prevalent fungi associated with maize kernels in many maize producing regions in the world (Yates et al., 1997). The ability

of this seedborne and soilborne fungus to affect germination, seedlings and subsequent disease development is controversial. Many authors claimed that *F. moniliforme* is an important seedborne pathogen of maize whereas other researchers reported that this fungus has no significant effect on the growth, development and yield of maize (van Wyk et al., 1988). Many studies have been conducted on pathogenic strains of *F. moniliforme* whereas a few investigations have been conducted on non-pathogenic strains. The first report on nonpathogenic *F. moniliforme* for its biocontrol ability to control *Fusarium* disease in gladioli dated back in 1980. The nonpathogenic *F. moniliforme* isolate M-685 demonstrated high levels of biocontrol activity against *Fusarium* rot (Magie, 1980). Since then, only one more report by van Wyk et al. (1988) has been published on the use of endophytic *F. moniliforme* for biological control of stem and ear rot disease in maize caused by *F. graminearum*. The authors demonstrated that pre-inoculation of maize with an isolate of *F. moniliforme* increased the fresh weights of seedlings while decreasing the stem and ear rot incidence. Studies on the biological control potential of *F. moniliforme* against plant parasitic nematodes in general or against *M. graminicola* in particular have not been investigated.

The biocontrol activity of non-pathogenic fungi toward plant parasitic nematodes is always linked to the colonization potential of the endophytic fungus used. Effective root colonization is also believed to be essential for biocontrol of fungal diseases (Handelsman and Stabb, 1996). Colonization of mutualistic *F. oxysporum* isolates in the host plants was reported to be important when direct effects of the antagonist on the target nematode have been detected and is suspected to be the mechanism responsible for nematode control (Niere, 2001; Vu, 2005 and Dababat and Sikora, 2007). The ability of mutualistic endophytic fungi to colonize the host plant therefore is important for their establishment, reproduction and survival and finally for their antagonistic activity (Speijer, 1993). The positive relationship between the level of colonization of *F. oxysporum* 162 in banana or tomato roots and their biocontrol activity against *Radopholus similis* (Vu, 2005; Mendoza, 2008) or *M. incognita* (Dababat and Sikora, 2007; Mendoza and Sikora, 2009) respectively, has been demonstrated. To achieve a high level of biocontrol, the endophytic fungi should be applied to the host plant for a sufficient period of time to facilitate their colonization, propagation and reproduction in

the endorhiza before host plant exposure to the nematode. In comparison, other authors elucidated that effective biocontrol is not necessarily connected with high rate of colonization (Niere, 2001). This may indicate that indirect mechanisms of action may also be involved in root-knot nematode control.

When applying biological control agents to plants, it is extremely important to ensure that they are not pathogenic to other rotation crops (Kerry and Evan, 1996). Although some *F. oxysporum* isolates are considered to be important antagonists to plant parasitic nematodes (Hallmann and Sikora, 1994; Sikora, 2003; Sikora et al., 2007), other strains are known to be important causal agents of *Fusarium* wilt disease (Olivian and Alabouvette, 1999; Olivian et al., 2003). There are only a few reports on the influence of non-pathogenic strains of *F. moniliforme* on crop plants (Magie, 1980; van Wyk et al., 1988) and none exist in rice. It is thus necessary to investigate the pathogenicity of the tested isolates before using them for biocontrol.

In addition, any positive effects of the multualistic fungi on the growth of the plant are of great interest. Some biological control agents such as mycorhiza, *Trichoderma* spp and *Fusarium* demonstrated growth promotion effects on the host plants (Niere et al. 1999; Pocasangre, 2000; Elsen et al., 2003). However, many others have been also reported to have neutral effects on the growth of host plant either in short or long term inoculation (Vu, 2005; Dababat, 2007).

The rice plants in some cropping systems like irrigated, lowland rain-fed or floating rice live most of their life under aquatic environments (Schalbroeck, 2001). The oxygen is brought down to the root tissue through the arenchyma tissue allowing rice plants to grow also under anoxic soil condition (Colmer, 2003). This special feature also enables some mutualistic endophytic microorganisms like fungi, bacteria as well as nematodes to survive over long periods of time under anoxic conditions (Verma et al., 2001; Bridge et al., 2005). Basically, if endophytic fungi demonstrate an ability to thrive in the rice root under flooded conditions, they should also interact negatively with pathogens and pest such as the root nematode.

The objectives of these experiments were to study the isolates Fe1 and Fe14 in relations to their:

1) colonization efficiency under oxic and anoxic soil conditions

2) colonization behaviour in different parts of the root

3) colonization over time

4) pathogenic potential

5) effects on rice growth

2. Experimental designs

2.1 Colonization under oxic and anoxic environments

The rice root-knot nematode is a sedentary endo-parasite which is highly adapted to both oxic (non-flooded) and anoxic (flooded) conditions. Therefore, it is strategically important to find antagonists that can establish and retain biocontrol activity in the rice root under anoxic soil conditions. In previous screening tests, the endophytic fungi *Fusarium moniliforme* isolates Fe1 and Fe14 demonstrated high levels of biocontrol against *M. graminicola* (Le et al., 2009). In the present study, the colonization of rice roots by Fe1 and Fe14 under oxic and anoxic soil conditions was investigated. The experiment was a two-way factorial design, consisting of fungal inoculated or non-inoculated rice treatments, with or without soil flooding following fungal treatment.

The mycelia and spores of Fe1 and Fe14 were coated onto 3-day old germinating seeds in a 2% methyl cellulose solution over a 2 hour period of agitation (See chapter 2). The coated seeds were then planted in experimental pots measuring 7x7x8 cm containing 300 g of sterilized soil (see chapter 2, 6). Rice plants were initially grown for 4 weeks under aerobic condition. After 4 weeks, half number of the pots were subjected to either flooding or non-flooding conditions for 2 more weeks. To quantify colonization by the fungus inside the root after this period of time, the roots were washed and surface sterilized in 1.5% NaOCl for 3 minutes followed by several rinses in sterilized tap water. Root systems were then cut into 1.5 cm long sections. Root pieces were placed on 10% PDA Petri plates and assessed for frequency of endophytic colonization. Fungal colonies growing out of the root pieces were identified based on morphological characteristics that clearly resembled the initial isolates used (Fe1 and Fe14).

2.2 Colonization in different root zones under oxic and anoxic conditions

The experiment was conducted with both isolates Fe1 and Fe14 using the same coating and inoculation procedures as described in section 2.1 of this chapter.

Four weeks after sowing, the roots were removed, sterilized and cut under aseptic conditions in 3 different parts: zone – 1 next to the root periphery, zone – 2 middle of

the root and zone 3 - near the stem base. For each zone, 10 root pieces of 1.5 cm length were cut and mounted onto two plates of 10% PDA. The presence of endophytic fungi growing out of the cut ends were analyzed in 2-day intervals for 14 days.

2.3 Root colonization of Fe14 over time under oxic conditions

This experiment was conducted to study colonization efficiency of Fe14 over time under oxic soil conditions. The fungal biomass was coated onto the germinating rice seeds and then the seeds were planted into the experimental pots as previously described (section 2.1). However, rice plants remained under oxic soil conditions until the end of the experimental period. The frequency of fungal colonization was assessed after 8, 10 and 12 weeks following the same experimental procedures as described in section 2.1 of this chapter.

2.4 Pathogenicity

Fe1 and Fe14 were tested for their pathogenicity on rice plant over a period of 5 months because different symptoms also develop later in the growth cycle of rice. The same seed coating technique as described in section 2.1 was applied in this experiment. Seeds coated with 2% methyl cellulose served as the control. Plants were grown for 5 months under greenhouse conditions and then harvested. The plants were examined weekly for disease symptoms. Pathogenic strains of *F. moniliforme* cause an economically important disease of rice, the bakanae disease. The common symptoms caused by this fungus such as foot rot, sheath rot were checked weekly for 20 weeks. Symptoms on panicle were not examined due to the longevity of the experiment under greenhouse conditions.

After harvesting, root and shoot weight and the length of stems were recorded. Fungal endophytic colonization was examined in the stem, leaves and roots. These tissues were surface disinfected in 0.5% NaOCl for 1 minute followed by several rinses in sterilized tap water. The sterilized stem or leaf sections of 1.5 cm were cut and mounted onto 10% PDA Petri dishes.

2.5 Effects on plant growth

The two endophytic fungi Fe1 and Fe14 increased the root and/or shoot weight of the rice plants in some earlier experiments. The effect on root weight and shoot weight were more obvious than on shoot heights. However, growth promotion effects of the two endophytic *Fusarium* isolates were not consistent. Therefore, growth promotion of the endophytes over short and long term time periods was investigated in glasshouse under both oxic and anoxic soil conditions.

The fungal biomass of either Fe1 or Fe14 was coated onto the germinating rice seeds in the same manner as in previous tests (see section 2.1). Inoculation with tap water served as the control. Four weeks after sowing, experimental plants were subjected to oxic and anoxic conditions. In the first period, the plants were harvested 2 weeks after flooding (i.e. 6 weeks after sowing) whereas in the second period, the experiment was terminated 8 weeks after flooding (i.e. 12 weeks after sowing). Root, shoot weights and the stem length were recorded. All stems and root systems were then dried in an oven at 65^{o}C for 48 hours. Dry root and shoot weight was also recorded. The experiment consisted of 2-way factorial design, with and without fungi under oxic or anoxic soil conditions and each treatment was replicated 7 times.

3. Results

3.1 Colonization under oxic and anoxic soil environments

Colonization of both isolates was very high under both soil water environments, ranging from 50 to 89% (Fig. 3.1). The recovery rate of the isolate Fe1 was slightly lower than that of the isolate Fe14 in both soil water regimes. The colonization rate of Fe14 ranged from 81% in non-flooded to 89% under flooded soil conditions whereas that of Fe1 was in the range of 50-60%. The isolate Fe14 was recovered in all roots of the treated plants (Data not shown). There was no evidence of colonization of the two *Fusarium* isolates in non-treated rice plants that served as controls.

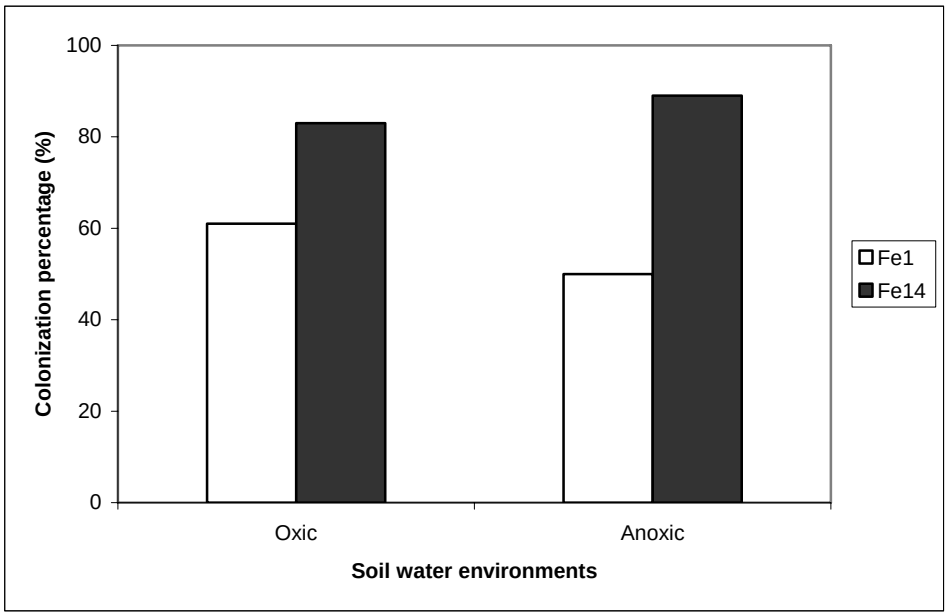

Figure 3.1: Colonization of the endophytic fungus *Fusarium moniliforme* isolates Fe1 and Fe14 in rice roots 6 weeks after fungal inoculation and 2 weeks after exposure to oxic or anoxic conditions (n=12).

3.2 Colonization in different root zones under oxic and anoxic soil conditions

Colonization efficiency of Fe1 and Fe14 in different root zones under oxic and anoxic soil conditions were investigated. In general, the colonization rate of Fe1 and Fe14 was very high, ranging from 78-93% (Table 3.1).

Table 3.1: Colonization efficiency of the endophytic *Fusarium moniliforme* isolates Fe1 and Fe14 in different root zones under oxic and anoxic soil conditions. Zone-1: next to the root periphery; zone-2: middle of the root; zone-3: near the culm base (n=12).

Root zone	Level of colonization (%)			
	Fe1		Fe14	
	Oxic	Anoxic	Oxic	Anoxic
Zone-1	91	83	93	91
Zone-2	88	90	83	82
Zone-3	86	83	88	78

Taking the soil water regime into account, there was no significant difference in colonization rate between the two isolates. Colonization was higher in zone-1 near the root tip. It was lower in the zone-2 middle of the root, and was lowest near the culm base, zone-3. However, these differences were not significant.

3.3 Level of colonization over time

Level of colonization of the isolate Fe14 over time was tested under oxic condition for a period of 3 months. Figure 3.2 showed that colonization decreased steadily over time. It was highest 8 weeks after inoculation with a recovery rate of 56%. The percentage of root colonization decreased 10 weeks after inoculation and was reduced to 27% after 12 weeks.

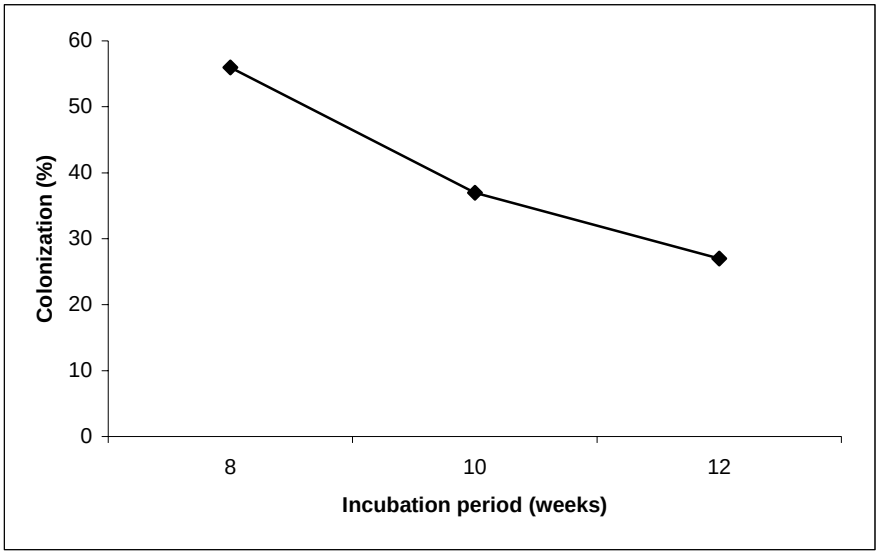

Figure 3.2: Level of root colonization of *Fusarium moniliforme* Fe14 in rice over 12 weeks under oxic soil environment (n=5).

3.4 Pathogenicity

The result showed that Fe1 and Fe14 did not alter the growth and development of rice and did not cause any disease symptoms. Inoculation with Fe1 and Fe14 resulted in slightly higher root weights and shoot height compared to those of the control plants. However, this difference was not significant among treatments. The fungal isolates were not recovered from the internal tissues of the stem nor the leaves. Typical symptoms associated with pathogenic strains of *F. moniliforme* (stunting or elongated seedlings) were not observed during the experimental time (Table 3.2).

Table 3.2: Influence of the endophytic fungus *Fusarium moniliforme* isolates Fe1 and Fe14 on the growth of rice and on the development of disease symptoms.

Treatments	Fresh shoot weight (g)	Fresh root weight (g)	Shoot length (cm)	Disease symptom on leave	Disease symptom on stem	Fungal recovery on root (%)
Fe1	11	10.4	41.2	nd	nd	24
Fe14	9.07	11.3	40.4	nd	nd	32
Control	8.25	11.7	38.8	nd	nd	0

(nd: not detected)

3.5 Effect on the rice growth

Inoculation of the two fungal isolates did not result in significant changes in growth of rice compared to that of the control. The fresh root weights of the treated plants were slightly higher than that of the non-treated plants under oxic soil conditions whereas growth was slightly lower in the fungal treated plants under anoxic conditions (Table 3.3). However, these differences were not significant. The fresh and dry shoot weights of the control plants were slightly higher under flooded conditions than those of fungal inoculated plants but again not significant. There was also no significant difference in the dry root and shoot weights of all treatments under the same oxic or anoxic conditions. Taking soil environment into account, the rice grown in anoxic conditions 6 weeks after sowing had significantly heavier roots and shoots than those grown in oxic soil. In comparison, there was no significant difference between each growth parameter under both soil water environments 12 weeks after sowing.

Table 3.3: Effect of the mutualistic fungi *Fusarium moniliforme* Fe1 and Fe14 on growth of rice

Treatment	Fresh root weight (g)		Fresh shoot weight (g)		Dry root weight (g)		Dry shoot weight (g)		Stem height (cm)	
	NF	F	NF	F	NF	F	NF	F	NF	F
6 weeks after sowing										
Fe1	0.97 ab	1.35 ab	0.90	1.24	0.09	0.12	0.19	0.22	32	37
Fe14	1.04 b	1.25 b	0.95	1.16	0.11	0.10	0.20	0.10	32	36
Control	0.66 a	1.81 a	0.86	1.49	0.09	0.23	0.18	0.26	31	38
P-value	$\leq$ 0.05	$\leq$ 0.05	ns	ns	ns	ns	ns	ns	ns	ns
12 weeks after sowing										
Fe1	2.68	3.54	2.37	2.13	0.58	0.50	0.52	1.06	30	39
Fe14	2.65	4.53	2.50	2.63	0.44	0.62	0.59	0.97	34	40
Control	2.64	2.29	2.81	2.60	0.64	1.32	0.46	0.56	33	33
P-value	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

(F: Flooding, NF: non-flooding; ns: not significant difference)

4. Discussion

4.1 Colonization under oxic and anoxic soil environments

The level of colonization of an antagonist on a host plant has been considered important for biocontrol efficacy against plant parasitic nematodes (Hallmann and Sikora, 1994). Many strains of non-pathogenic *Fusarium oxysporum* have been found to reduce nematode infection on banana (Niere et al., 1999, Pocasangre, 2000; Vu, 2000; zum Felde et al., 2006; Mendoza, 2008) and tomato (Dababat and Sikora, 2007, Sikora et al., 2007). Colonization of endophytic fungi is believed to be important for biocontrol efficacy, especially when direct effects of the antagonists on the target organism have been detected (Niere, 2001; Vu, 2005). Dababat and Sikora (2007) demonstrated that the endophytic mutualistic *Fusarium oxysporum* isolate Fo162 successfully colonized the endorhiza of tomato plants, including *Fusarium* wilt resistant varieties and level of colonization was closely related to biocontrol. Similarly, Vu (2005) reported high level of colonization of Fo162 along with high level of biocontrol against the burrowing nematode *Radopholus similis* on banana.

In the present experiment, colonization of both *Fusarium* isolates Fe1 and Fe14 was very high regardless of the soil conditions. Fungal endophytes, including *Fusarium* spp. have been isolated from rice (Fisher and Petrini, 1992; Tian et al., 2004; Vallino et al, 2009). This endophytic community is believed to have antagonistic potential against plant pests and pathogens. However, studies on the colonization of these beneficial fungi in rice under both oxic and anoxic conditions are non-existent. In the present study it was shown for the first time that a fungal endophyte is capable of surviving for a long period of time in rice under anaerobic conditions. The results proved the hypothesis that the arenchyma tissue translocated oxygen in the roots under anoxic conditions that can support potentially beneficial aerobic organisms such as endophytic fungi (Verma et al., 2001).

The level of colonization of the two endophytic isolates Fe1 and Fe14 suggests that these two fungi might be able to compete antagonistically with the rice root-knot nematode once they are trapped together in the root tissue under anoxic soil conditions.

This finding is important because it might lead to high biocontrol activity under anoxic soil condition when no other control measures are available.

4.2 Colonization in different root zones under oxic and anoxic conditions

The high colonization rate in all parts of the root system under both oxic and anoxic conditions demonstrated the ability of these two isolates to survive in the rice root under anaerobic conditions. Interestingly a slightly higher rate of colonization was observed near the root tips. The results are similar to the findings reported by Olivian and Alabouvette (1997). In an experiment conducted on tomato using a non-pathogenic strain of *Fusarium oxysporum*, colonization was mainly observed in the root hairs 24 hours after the fungal inoculation. After that, there was no preferential zone for colonization. The higher colonization rate of the fungi just behind the root tips is an important finding because the root section just behind the root tip is usually the zone of nematode penetration (Bridge et al., 2005).

The results suggested the possibility of a direct effect of the endophytic fungi on the nematode early in the disease cycle or competition for space between the endophytes and the nematode inside the root.

4.3 Colonization of Fe14 over time

Colonization of the endophyte Fe14 was high but decreased steadily over time after the intial fungal application. The same tendency was obtained by Dababat and Sikora (2007) when they studied the colonization behaviour of the mutualistic fungus *F. oxysporum* Fo162 in tomato. They demonstrated that recovery of the endophyte decreased over time eventhough antagonism of the fungus against the root-knot nematode *M. incognita* in tomato was still active. Similar results were reported by Niere (2001) who re-isolated *F. oxysporum* 1 and 5 months after fungal inoculation from banana plants and observed that colonization decreased significantly after 5 months. In contrast, Mendoza (2008) reported that the colonization rate of Fo162 increased with time when inoculated to banana plants in greenhouse trials. Vu (2005) also reported very high colonization rates of 4 endophytic *Fusarium oxysporum* isolates on banana after 14 weeks of inoculation. The level of endophytic colonization of the *Fusarium*

depended on the fungal strain used and was affected by banana cultivars used (Speijer, 1993; Hallmann and Sikora, 1994; Pocasangre, 2000; Niere et al., 1999; Vu, 2005; zum Felde et al., 2006).

As previously discussed, colonization potential of the endophytic fungi was considered to be important when direct effects of the fungi on the nematode or pathogen were detected (Alabouvette et al., 2001; Niere, 2001). On the other hand, it was also elucidated that high levels of colonization did not always indicate high biocontrol efficacy (Niere, 2001). In this case, other indirect mechanisms of action are involved instead of the direct effect of the antagonist on the target pathogen. The present study is the first to show that a non-pathogenic and mutualistic antagonist, Fe14 establised well in the rice root but that colonization decreased slowly over time.

4.4 Pathogenicity

Fusarium is abundant in soil ecosystems. This genus is also frequently detected in the plant tissues in almost all crops and regions. Many species are important pathogens in agriculture such as *F. oxysporum*, *F. solani*, *F moniliforme*. However, some of them also live asymptomatically inside plant roots. Determining pathogenicity of a potential biological control agent is an important step before applying such an agent to a crop.

The fungal disease caused by *F. moniliforme* in rice is often referred as foot rot or bakanae disease. This disease can be observed in the seedbed or in the field. The symptoms may appear in the seedling stage with abnormal elongation or stunting of the stem or in later stage empty panicles. The rice plants may also turn yellow during the vegetative stage (Mew and Gonzales, 2002).

Long term study of the two isolates Fe1 and Fe14 in rice showed that these isolates were not pathogenic to rice. Typical disease symptoms on the root, stem or grain never appeared. Moreover, plant growth and development was also not affected when the isolates were introduced to rice. In addition, the fungi were not recovered in the stem and leaf tissues. It was stated that pathogenic strains usually colonize all parts of maize such as stem, leaf and grain (Yates et al., 1997, Bacon et al., 2000). Since the endophytes Fe1 and Fe14 never produced disease symptoms on rice, their absence in

the stem and leaves were also expected. Therefore, it could be concluded that the two isolates Fe1 and Fe14 were not pathogenic to rice.

4.5 Effect of endophytic fungi on the growth of rice

The effect of biological control agents on plant health is important especially of seedlings because it has an impact on yield over time. It is also desirable to have a biocontrol agent that can effectively promote the growth of the seedling making it more tolerant to nematode infection.

In the present study, inoculation of rice plants with the fungal endophytes slightly increased the root and shoot weights under non-flooded conditions while slightly decreasing plant weights under flooded conditions. Overall, there was no significant difference amongst treatments at the same soil water condition, either in short term or long term studies.

Some biological control agents have been reported to enhance plant growth in different plants. Non-pathogenic *Fusarium* strains promoted banana growth in different banana cultivars (Niere et al., 1999; Pocasangre, 2000; Vu, 2005; zum Felde et al., 2006, Mendoza, 2008). Moreover, growth promotion effect of the biocontrol agents on host plants were reported on AMF (Elsen et al., 2003) and endophytic *Trichoderma* (zum Felde, 2006) on banana. In comparison, endophytic fungi having antagonistic potential may not influence the growth of the host plant. Dababat (2007) observed no growth effect of Fo162 on tomato plants even though this fungus caused high levels of biocontrol against *M. incognita*. This type of effect is also expected as the biocontrol agents were first selected according to their biocontrol activity and not for growth promotion.

There was however an interaction between growth parameters of rice and the soil water environments as seen in significant increases of growth under oxic soil conditions. This result was expected since BR11 obtained from Bangladesh is an irrigated variety.

5. Conclusion

In this chapter, the ability of *F. moniliforme* isolates Fe1 and Fe14 to colonize the endorhiza of rice and influence the growth of rice under oxic and anoxic environment was studied. Pathogenicity also was investigated. Based on the results the following conclusions can be drawn:

1) *F. moniliforme* isolates Fe1 and Fe14 have a high capacity to colonize the endorhiza of rice under both oxic and anoxic soil conditions especially during the important seedling stages of growth

2) Fe1 and Fe14 colonized well in different root zones with colonization slightly higher in the zone behind the root tip which is important for direct interaction at the site of J2 penetration

3) High levels of colonization of Fe14 persisted over time and therefore can have activity to many life cycles of *M. graminicola*

4) Both isolates showed no bakanae disease symptoms over long periods of time proving the non-pathogenicity of the strains

5) Inoculation of Fe1 and Fe14 did not result in significant influence on rice growth

CHAPTER 4: Modes of action of endophytic *Fusarium moniliforme* Fe14 toward *Meloidogyne graminicola* in rice

1. Introduction

The use of micro-organisms in controlling plant pests and diseases including plant parasitic nematodes has become an important alternative to chemical and traditional cultural practices, especially in regions where these control measures are not suitable such as in monocultured rice production systems. Extensive research has been carried out on biological control of nematodes in the last two decades. The reasons for this shift are: 1) the lack of resistance, 2) shorter rotations and 3) toxicity of nematicides to many non-target living organisms and their high cost. In many non-cash crops like rice effective control measures are not adaptable.

Plant parasitic nematodes have many natural enemies such as insects, viruses, fungi and bacteria. The source of antagonists may come from the soil, plant tissues or even from the nematode body and eggs. However, the soil, being a rich ecosystem with millions of bacteria and fungi, remained until recently the most important source of antagonists. Now stress is being placed on rhizosphere and endophytic plant habitats for novel antagonists.

Mode of action studies are one of the most important aspects of biological control research because it helps to understand the biology of the pest or disease and the infection processes through which weak links could be broken to favour biocontrol. Biochemical analysis and genetic aspects also are important for breeding, transgenic development and detection of specific chemical compounds for control. Antagonists have different mechanisms that act against plant parasitic nematodes including: predation, parasitism, pathogenesis, competion, repellence or induced systemic resistance (Stirling, 1991; Sikora, 1992; Hallmann and Sikora, 1994; Hasky-Günther and Sikora, 1995; Schuster et al., 1995; Kerry, 2000; Reitz et al., 2001; Reitz and Sikora, 2001, Diedhiou et al., 2003; Fravel et al., 2003; Vu et al; 2006; Dababat and Sikora, 2007; Padgham and Sikora, 2007; Rumbos et al., 2006; Sikora et al., 2007; Elsen et al., 2008;). Some fungi such as *Trichoderma* or *Paecilomyces* spp. produce

toxins that kill or inhibit the development of the eggs, prevent egg hatching or are toxic to the nematode after hatching (Jatala et al., 1980; Dube and Smart, 1987; Kiewnick and Sikora, 2006; Siddiqui et al., 2000; Khan et al., 2001; Mendoza et al., 2006; Kiewnick, 2009). Plants can also activate protective mechanisms upon contact with microorganisms and through induced or aquired resistance to reduce pest attack (Vu et al., 2006; Dababat and Sikora, 2007a).

Scientists working on biological control of plant parasite nematodes now consider fungal endophytes potentially important due to microbial-plant interactions that may have direct and indirect control activity. Endophytes are microorganisms that reside inside plant tissues that have either multualistic, pathogenic or non-pathogenic effects on plant development and health. Some of these organisms, especially endophytic fungi have been well studied. For example, biocontrol potential of the mutualistic *Fusarium oxysporum* Fo162 against a range of plant parasitic nematodes has been extensively studied on different crops such as banana, tomato, pepper, melon and squash under greenhouse conditions (Vu, 2005; Dababat and Sikora, 2007b; Mendoza and Sikora, 2008; Mehjivar and Sikora, 2010). Antagonistic activity of endophytes against several important nematodes like *M. incognita* and *R. similis* expressed through different modes of action such as interference with juvenile pentration, repellence through alteration of root exudates or lack of attraction. Some of these mechanisms are triggered by induction of systemic resistance (Hallmann and Sikora, 1998; Vu et al., 2006; Dababat and Sikora, 2007a).

Only a limited number of investigations have been carried out on the mechanisms of biological control of the rice root-knot nematode *M. graminicola* with microbial endophytes. The endophytic bacterium *Bacillus megaterium* Ni5SO11 isolated from rice roots demonstrated high levels of biocontrol against *M. graminicola* in rice through prevention of penetration and the production of toxic metabolites that inhibited egg hatching and juvenile mobility (Padgham and Sikora, 2007). However, the mode of action of antagonistic endophytic fungi toward the rice root-knot nematode have not been studied. Therefore, understanding the mode of action of endophytic fungal antagonists, their behaviour in different environments and interaction with other living

organisms is the key to success in biocontrol of the rice root-knot nematode. The objectives of the following research conducted with Fe14 were to investigate:

1) efects on juvenile penetration

2) existent of induced systemic resistance

3) repellent activity and effects on nematode mobility

4) influences of the endophyte on nematode development

5) effects of the mutualistic fungus on nematode reproduction

6) biocontrol activity under oxic and anoxic soil environments

2. Experimental design

General methods are presented in chapter 2. Only specific changes in design are given in this chapter.

2.1 Juvenile penetration

In this experiment, combining endophyte seed coating and soil drenching methods with the endophyte was applied to test for compatibility. Fungal biomass of Fe14 was coated onto the pre-germinated rice seeds using 2% methyl cellulose at a rate of 10^6 spores per seed at sowing. Rice seeds coated with only methyl cellulose served as the control. (See chapter 2). The coated seeds were planted in experimental pots measuring 7x7x8 cm filled with 300 g of autoclaved sandy soil. Three weeks later, additional 5 ml of fungal suspension containing 5x 10^7 spores were drenched into 3 holes made around the root system of each rice seedling. One week after the second fungal inoculation, 2 ml of tap water containing 1000 J2 were inoculated in the rhizosphere of each plant. The experiment was harvested 2 weeks after the nematode inoculations. All plants were uprooted and washed. Fresh root and shoot weights were recorded and then the roots were stained with 1% Fuchsine acid. The stained roots were cut into small pieces and macerated in a commercial blender for 3 minutes (See chapter 2). The root suspension was made up to 100 ml and a 10 ml aliquot was taken to count the number of penetrated nematodes. The experiment was conducted twice.

2.2 Induced systemic resistance

A split-root experiment was designed to study the presence of induced systemic resistance. Rice seeds were surface sterilized and pre-germinated on wet filter paper for 3 days (See chapter 2). Germinating rice seeds were then sown in the experimental pots filled with 300 g autoclaved sandy soil. Five weeks after sowing, rice seedlings were uprooted and washed carefully. Each root system was split into two equal parts. Each half was put through a hole in the bottom of an experimental pot and planted into 2 separate pots placed at the bottom (Fig. 4.1). The roots in the two bottom pots were spatially separated and covered with soil and then individually treated. One week after

root splitting, the roots growing in one pot of the experimental apparatus were inoculated with $5x10^6$ spores of *F. moniliforme* strain Fe14 by drenching 5 ml of fungal suspension into 3 holes made around the root system. This pot is called the Inducer side. The other half of the root system of the same apparatus was treated with water and is called the Responder side. Plants with both root halves treated with water served as controls. Fungal inoculation was repeated 2 weeks later in the Inducer side while tap water was added to the Responder side and also to the control plants.

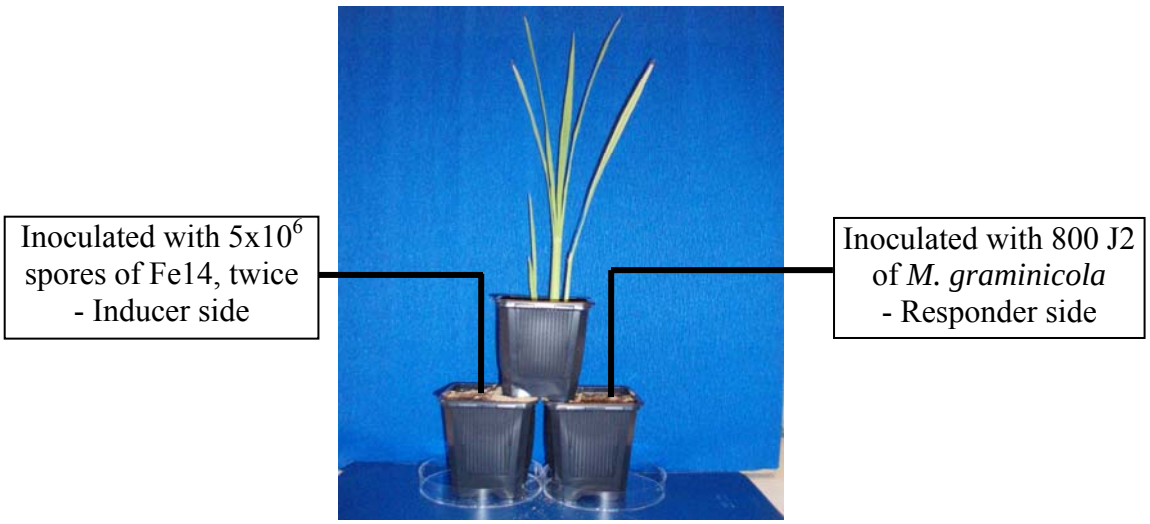

Figure 4.1: Design of split-root apparatus used to determine *Fusarium moniliforme* Fe14 induced systemic resistance against *Meloidogyne graminicola* in rice.

One week after the second fungal inoculation, 800 J2 of *M. graminicola* were added to the Responder side of each system. The experiment consisted of two treatments: 1) Inducer side treated with water and Responder side inoculated with *M. graminicola*, and 2) Inducer side inoculated with Fe14 and Responder side inoculated with *M. graminicola*.

The experiment was terminated 3 weeks after nematode inoculation. Each experimental apparatus was carefully washed to collect all roots in the two pots separately. Gall number was determined in the Responder side. After counting the galls, colonization of Fe14 was examined in both root systems. The experiment consisted of 6 replicates and was conducted twice. However, in the second experiment, rice plants were grown for 6

weeks before splitting the rice root due to a longer period needed for plant growth caused by cooler temperature in the greenhouse.

2.3 Repellent effect of the root exudates

Rice seeds were surface sterilized, pre-germinated and then coated with fungal biomass of Fe14 as previously described (Chapter 2). Seeds coated with only methyl cellulose served as controls. Endophyte treated rice seeds were planted in experimental pots and additional fungal spores were inoculated 3 weeks later at a rate of 10^5 spores per gram soil. Control plants were treated with tap water. The rice seedlings were grown under greenhouse conditions for 4 weeks where they were watered daily and fertilized weekly with Yoshida solution. To collect root exudates, rice plants were not watered for 2 days to keep them just below the permanent wilting stage. Then 200 ml of tap water were added to each pot and the water was allowed to percolate through the soil and out of the bottom holes into the trays placed underneath. The root exudates were collected in separate bottles for the control plants and for Fe14 treated plants. In total, there was about 600 ml of root exudate solution collected from each treatment. The two exudate solutions were filtered through Whatman paper (Schleicher & Schuell MicroScience-Germany).

Small plastic chambers measuring 12x2x2 cm (figure 4.2) were filled with approximately 80 g of sterilized fine sand which was obtained by sieving sand through a sieve with an aperture of 250 µm and then autoclaved. The sand was moistened with sterilized tap water to facilitate nematode movement. The experiment was designed as followed: 1) both sides inoculated with exudates from control plants; 2) both sides inoculated with root exudates from Fe14 treated plants; 3) one side with root exudates from Fe14 treated plants, one side with root exudates from control plants; 4) the absolute control, both sides with tap water. A 1 ml suspension containing 1000 J2 was pipetted onto the middle of the chamber and 1 ml of the root exudates was drenched onto each side of the chamber arm. A plastic cover was gently place on top of the chamber and the two ends were carefully wrapped with parafilm to prevent moisture loss. One ml of sterilized tap water was added daily to each chamber end to maintain a stable moisture level. All plastic chambers were placed in the greenhouse at a mean

temperature of 28°C for 7 days. For evaluation, the chambers were gently opened and the sand in each 1.5 cm section from the middle was carefully collected into a 50 ml test tube filled with 20 ml tap water. The number of the nematodes in each tube was determined in sub samples of 2 ml aliquot after stirring and setting out of the sand. This experiment was conduced twice and each treatment was replicated 6 times.

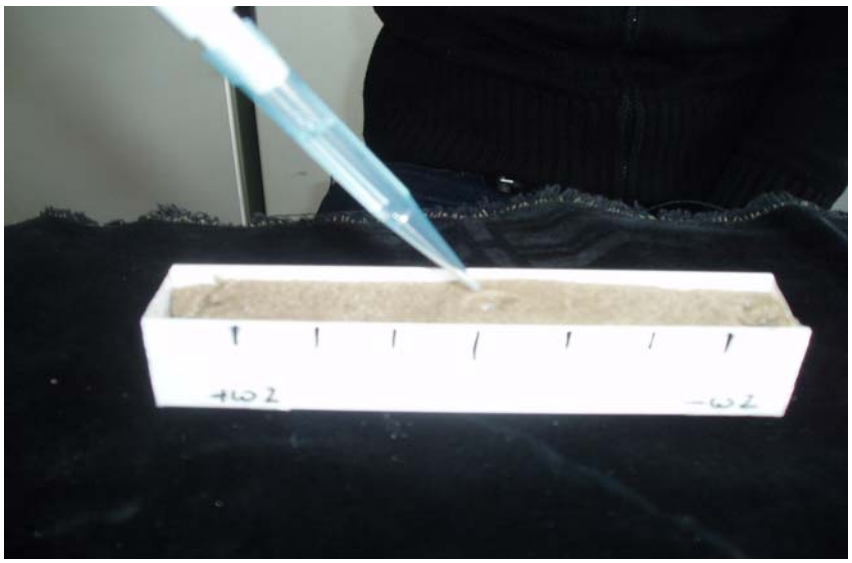

Figure 4.2: Design of the plastic chamber used to investigate the influence of root exudates from rice treated with *Fusarium moniliforme* Fe14 on *Meloidogyne graminicola* movement.

2.4 Nematode development and reproduction

A series of experiments were conducted to investigate the influence of Fe14 on the development and reproduction of *M. graminicola* after penetration. The speed of nematode development in different life stages, the male to female ratio and the reproduction capacity of the female were studied.

In the first set of experiments on development, combined seed treatment and soil drench method was used for inoculation of Fe14 as previously described in section 2.1 of this chapter. One week after the second fungal inoculation, 2 ml of a suspension containing 1000 J2 was added into 3 holes made around the root of each rice seedling. The nematodes were allowed to penetrate the rice roots for 3 days to synchronize their development. All rice seedlings were then uprooted and washed carefully and then

transplanted into new experimental pots filled with 300 g of sterilized sandy soil. Rice seedlings were watered daily and fertilized weekly with Yoshida solution. The experiment was terminated 6 weeks after nematode inoculation. All roots were washed and stained with 1% Fuchsine acid and then stored at 4^{o}C. For evaluation of the nematode development, the roots were cut into small pieces and macerated to expose the nematodes in the root cortex. The number of nematodes in 3 stages: juveniles, female and male were counted under microscopes. The experiment conducted twice with 7 replicates in experiment 1 and 11 replicates in experiment 2.

Reproduction experiments followed the same procedure as described above in which nematode development was synchronized. The experiment was harvested 4 weeks after nematode inoculation. The roots were weighed and then stained with 1% Fuchsine acid. The stained roots were examined under a stereomicroscope and single females were removed from the root with tweezers and then placed in a cavity slide (d=4 cm) containing 5 ml of 0.1 M NaCl solution. Fifteen females were randomly selected per root system. The females were then gently crushed using a small needle and the suspension containing eggs in 0.1 M NaCl was vortexed for 3-5 seconds to open the egg sacs. The average number of eggs per female was determined in a sub sample of 1 ml egg suspension.

2.5 Biological control activity under oxic and anoxic soil conditions

Rice seeds were surface sterilized and then pre-germinated as described in section 4 of chapter 2. The fungal isolate Fe14 was inoculated twice to rice seedlings as described in the previous section. One week after the second fungal inoculation, 1000 J2 of the root-knot nematode were inoculated to each rice seedling. Three days later, nematode development was synchronized by uprooting plants, washing them very carefully and transferring to new experimental pots which were divided into two parts. In the flooded part, the experimental pots were slowly submerged in water for 3 days prior to transplanting to allow for anaerobic conditions. The non-flooded part was kept under aerobic conditions. Eight weeks after transplanting, the experiment was terminated. All plants were uprooted and washed carefully. Fresh root and shoot weights were recorded and then roots were stained with 1% Fuchsine acid. The stained roots were cut into

small pieces and macerated by a commercial blender to expose nematodes from the root cortex. The root suspension was made up to 100 ml and a 10 ml aliquot was taken to count the number of nematodes inside the root.

3. Results

3.1 Juvenile penetration

Inoculation of rice seedlings with the endophytic fungus *F. moniliforme* Fe14 4 weeks in advance of *M. graminicola* introduction resulted in a significant 55% reduction in penetration of the nematode (Fig 4.3).

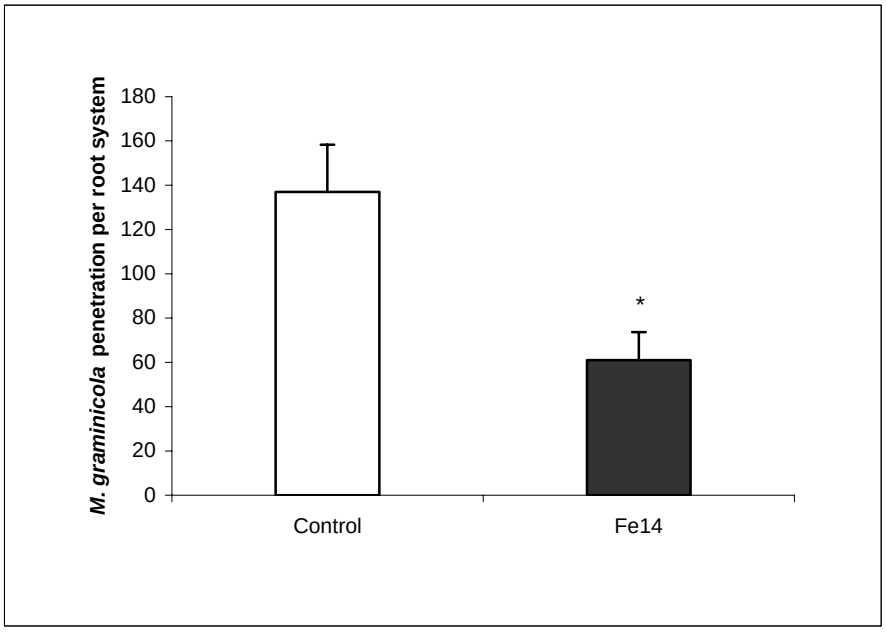

Figure 4.3: Influence of *Fusarium moniliforme* isolate Fe14 on *Meloidogyne graminicola* penetration of rice when the endophyte was allowed to establish 4 weeks before nematode inoculation. * = significantly different from the control based on the T-test ($P \leq 0.05$; n=14).

3.2 Induced systemic resistance

The ability of Fe14 to induce systemic resistance was investigated in this study by applying the fungus to the Inducer side and inoculating *M. graminicola* in the Responder side of a split-root rice plant. Application of Fe14 to the Inducer side significantly reduced nematode invasion to the Responder side.

In the first experiment, there was a large decrease of up to 60% whereas in the second experiment the reduction reached 38% (Fig. 4.5).

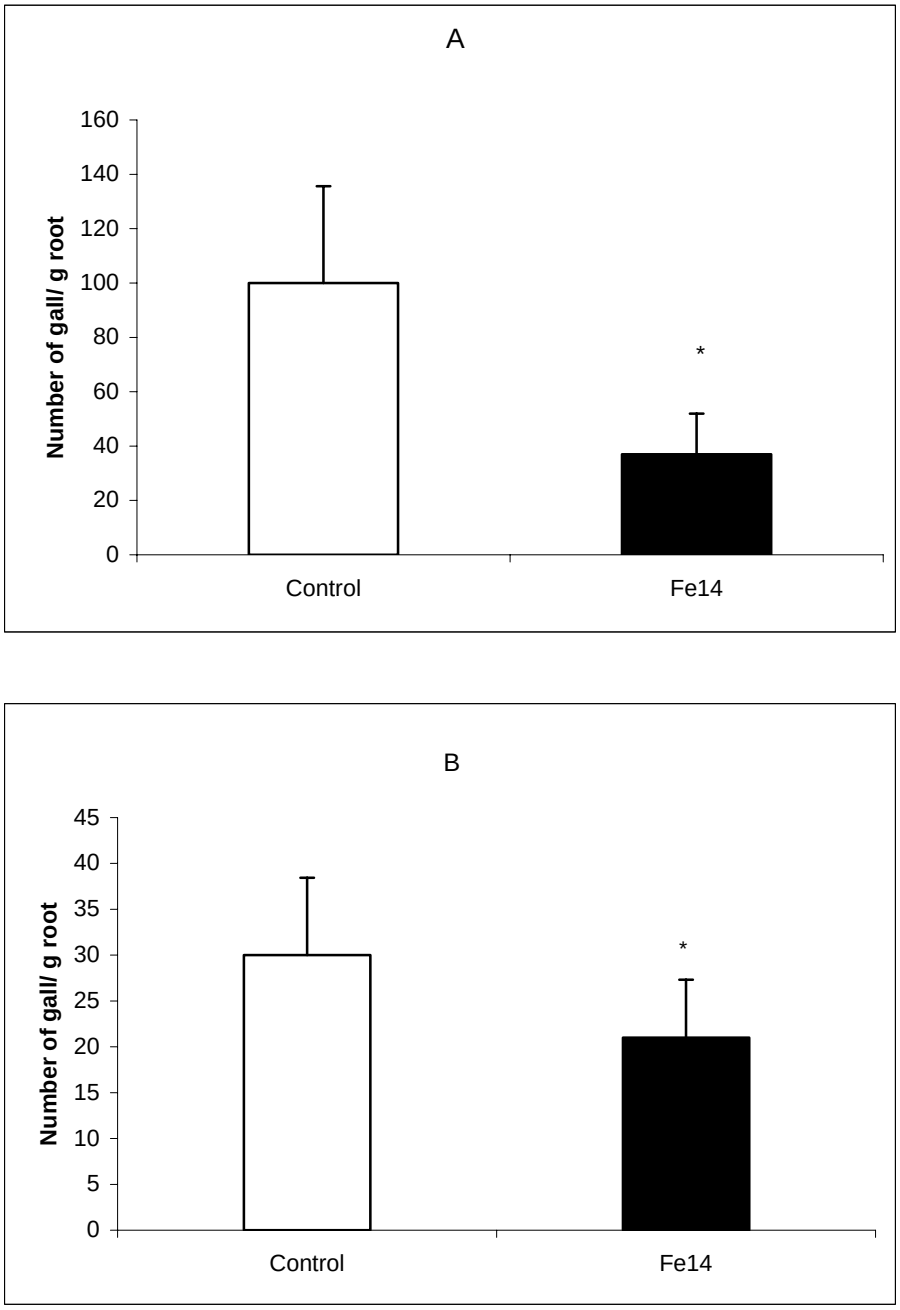

Figure 4.4: Influence of *Fusarium moniliforme* Fe14 inoculation to the Inducer side on *M. graminicola* penetration in the Responder side of a rice split-root system. A and B are the first and the second experiments respectively. * = significantly different from the control based on the T-test ($P \leq 0.05$, n=7). Bars represented standard errors of the mean.

3.3 Repellent effect of root exudates

One week after nematode inoculation in the middle of the chamber, the movement of the nematode toward the two sides of the plastic chamber was significantly affected by root exudates from differently treated plants. The number of nematodes that moved to the side treated with root exudates from Fe14 treated plants was significantly lower by 46-62% in the sections of 1.5 – 3.0 cm to 4.5-6.0 cm from the middle point compared to the number of nematodes that moved to the side treated with root exudates from control plants. The number of nematodes that remained in the middle part was not significantly different between the two sides of the test chamber (Fig. 4.6).

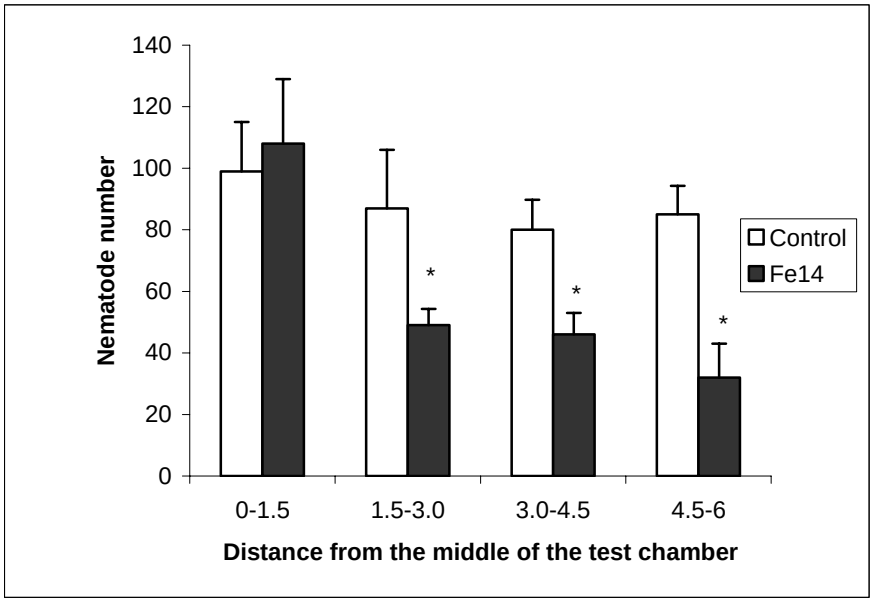

Figure 4.5: Effect of *Fusarium moniliforme* Fe14 root exudates (Fe14) and root exudates from control plants on *Meloidogyne graminicola* movement in fine sand after 7 days. Means with * are significantly different within one set of columns based on the T test ($P \leq 0.05$; n=12). Bars represented standard errors of the mean.

There were no significant differences in nematode movement when both sides of the chamber were inoculated with root exudates from Fe14, exudates from uninoculated plants or with only water (Fig. 4.7)

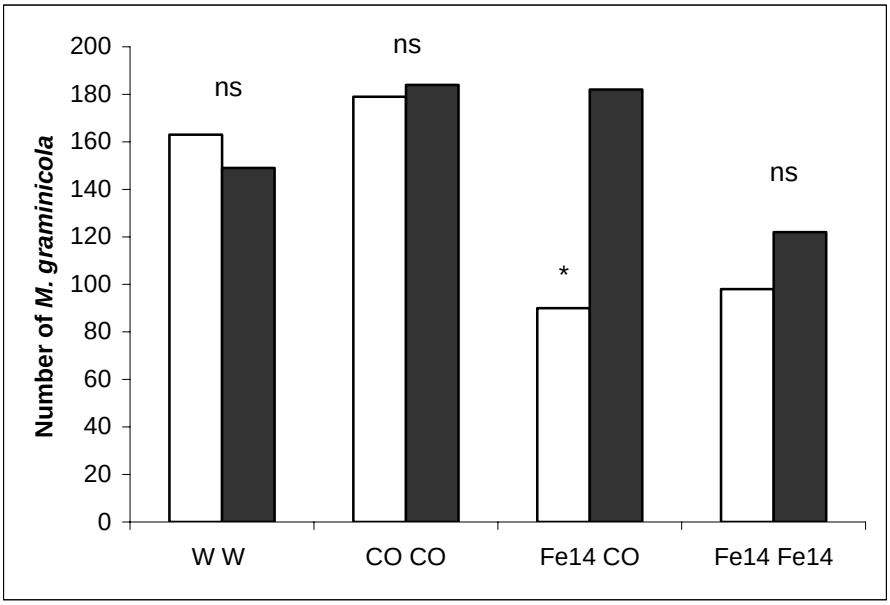

Figure 4.6: Effect of Fusarium *moniliforme* Fe14 root exudates (Fe14); root exudates from control plant (CO) and water (W) on migration of *Meloidogyne graminicola* in fine sand after 7 days. Means with * are significantly different within one set of columns based on the T-test ($P \leq 0.05$; n=12).

3.4 Nematode development and reproduction

Treatment of rice plants with Fe14 altered the number of female and male inside the root. The number of males in vermiform was significantly higher in the roots treated with Fe14 when compared to that of the control. The number of females on the contrary was lower in roots of Fe14 treated plant. The male to female ratio in the Fe14 treated plant was much higher than that in the control plants (Fig. 4.8A).

When the experiment was repeated, males in vermiform were not observed but the number of female was also significantly lower in the Fe14 treated plants compared to the control treatment (Fig. 4.8B).

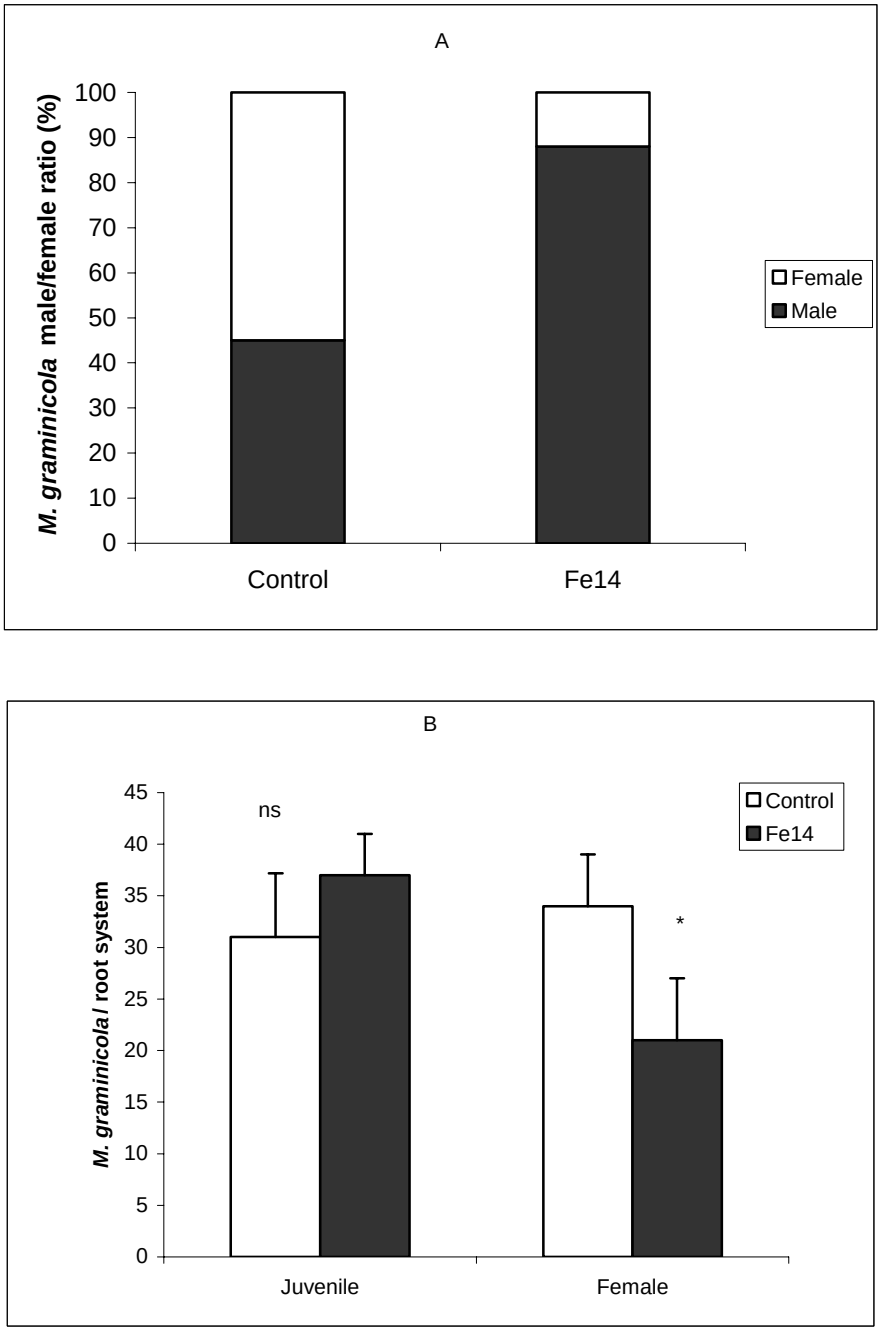

Figure 4.7: Influence of *Fusarium moniliforme* Fe14 on development of *Meloidogyne graminicola* in rice root 6 weeks after synchronized nematode infestation. A: male to female ratio; B: number of juveniles and females. Columns with * indicate significant difference from the control based on the T-test, ns: not significant. Bars represented standard errors of the mean.

Colonization of rice root by Fe14 influenced nematode female fecundity by reducing the number of eggs per female by 30% compared to that of control plant. However the reduction was not significantly different.

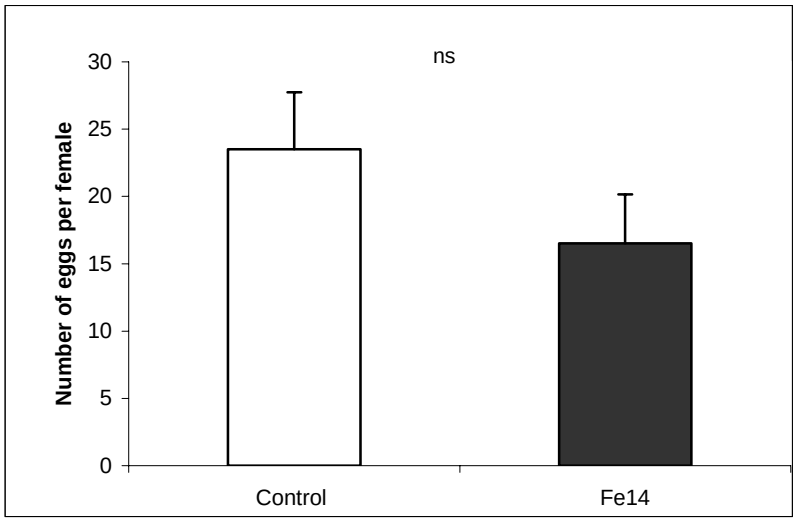

Figure 4.8: Number of eggs per female of *Meloidogyne graminicola* in rice roots treated with the endophyte *Fusarium moniliforme* Fe14. ns: not significant difference based on T-test ($P \leq 0.05$, n=5).

3.5 Biological control activity under oxic and anoxic soil conditions

Biocontrol caused by Fe14 was very high under both soil water environments (Fig. 4.10). Under oxic soil conditions, Fe14 significantly reduced nematode population in the rice endorhiza by 60%. The level of biocontrol of Fe14 was slightly lower under anoxic soil conditions but it was still significantly different to the control. In general, nematode population inside the root was smaller under anoxic soil conditions compared to oxic soil environments.

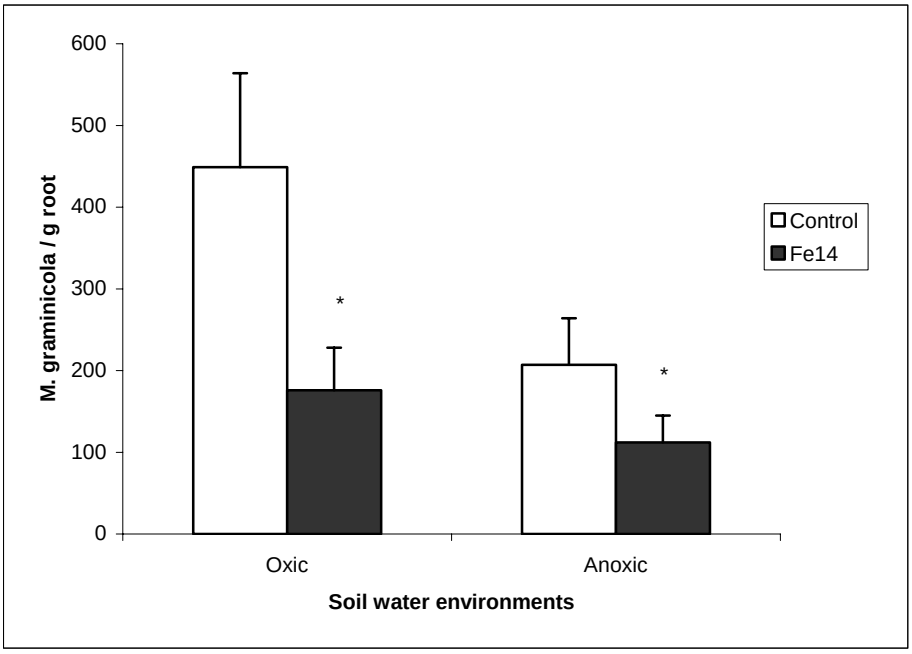

Figure 4.9: Effect of colonization of the non-pathogenic endophytic *Fusarium moniliforme* isolate Fe14 on the root-knot nematode *Meloidogyne graminicola* population in rice 8 weeks after nematode inoculation under oxic and anoxic soil environments. Means with * are significantly different within one set of columns based on the T-test ($p \leq 0.05$, n=10).

4. Discussion

4.1 Effect of Fe14 on the nematode penetration

The mutualistic endophyte *Fusarium moniliforme* isolate Fe14 significantly reduced nematode penetration into the rice root. Penetration of the nematode was reduced significantly by 55% two weeks after inoculation when the fungus colonized the rice plants. The results demonstrated high level of early protection of the seedling against the nematode mediated by the endophytic fungus. This is the first time that an endophytic fungi have been shown to be antagonistic to the root-knot nematode in rice. However, other researchers have shown that the endophytic fungi can reduce nematode penetration in other crops (Hallmann and Sikora, 1994a, b; Niere et al., 1999; Pocasangre, 2000; Sankaranarayana et al., 2001; Dieuhiou et al., 2003; zum Felde et al., 2004; Vu, 2005; Dababat, 2007). For instance, the mutualistic endophyte *Fusarium oxysporum* Fo162 reduced penetration of *Meloidogyne incognita* in tomato and *Radopholus similis* in banana by 28% to 41% respectively (Vu, 2005; Dababat, 2007). Many studies have elucidated that fungal endophytes may alter chemical or physical properties of the root exudates or interact with the plants to produce chemical or hormone complex compounds which repel or interfere with nematode attraction (Diez and Dusenbury, 1989; Viljoen et al. 2006; Dababat and Sikora, 2007). The mechanism by which the fungus reduces nematode penetration will be discussed in more detail in the following sections.

4.2 Induced systemic resistance

In split-root experiments, Fe14 demonstrated induced systemic resistance to *M. graminicola*. The application of Fe14 to one side of the split-root system resulted in a significant reduction of *M. graminicola* infestation in the other side. This clearly shows that protection by Fe14 is the result of a mode of action coupled with induced systemic resistance to the root-knot nematode in rice.

Induced systemic resistance (ISR) is commonly defined as “a phenomenon whereby resistance to infectious disease is systemically induced by localized infection or

treatment with microbial components or products or by a diverse group of structurally unrelated organic and inorganic compounds. The activity of the inducing agents is not due to antimicrobial activity *per se* or their ability to be transformed into antimicrobial agents. However, antimicrobial agents can induce resistance, and they provide protection from the time of application until ISR is fully expressed" (Kuc', 2000). ISR can be triggered by exogenous application of virulent or avirulent pathogens, plant growth promoting rhizobacteria and various chemicals including salicylic acid, jasmonic acid, benzothiadiazole-7-carbothioc acid S-methyl ester (BTH), 2,6-dichloro isonicotinic acid (DCINA) and β-aminobutyric acid (BABA) (Kuc, 2001; Oka and Cohen, 2001; Ramamoorthy et al., 2001). ISR can be local or systemic when the entire plant becomes protected against later infections (van Loon, 1997).

Induced systemic resistance by non-pathogenic micro-organisms has been demonstrated against fungi, bacteria and viruses in a wide range of plants such as *Arabidopsis*, bean, cucumber, radish and tomato (in review paper by van Loon et al., 1998). ISR was also reported for non-pathogenic *Fusarium oxysporum* isolates against *Fusarium* wilt disease on several vegetable crops (Mandeel and Barker, 1991; Alabouvette et al., 1993; Fuchs et al., 1997). For example, Fuchs et al. (1997) used the non-pathogenic *F. oxysporum* strain Fo47 to control *Fusarium* wilt incidence in tomato and demonstrated the presence of an induced systemic resistance signal.

In biocontrol of plant parasitic nematodes, ISR has been frequently reported. This mechanism was first found when using rhizobacteria in potato and tomato (Hansky-Günther et al., 1998; Reitz et al., 2000, 2001; Munif et al., 2001; Hauschild et al., 2004). Later, Siddiqui and Shaukat (2002, 2004) also reported systemic resistance induced by the rhizobacteria *Pseudomonas aeruginosa* and *P. influorescens* against the root-knot nematode *M. incognita* on tomato. Moreover, ISR was reported with the arbuscular mycorrhizal fungi (AMF) in bioprotection of banana against the burrowing nematode *R. similis* (Elsen et al., 2008) or with bacteria for biocontrol of several plant parasitic nematodes (van Loon et al., 1998; Reitz and Sikora, 2001; Mwangi et al., 2002; Siddiqui and Shaukat, 2004).

Recently, ISR has been demonstrated to be the main component of the overall mechanism of action of mutualistic fungal endophytes involved in biocontrol of plant parasitic nematodes. Vu et al. (2006) demonstrated that inoculation of the mutualistic endophyte *F. oxysporum* Fo162 to one side of a banana split-root system led to the reduction of *Radopholus similis* infection in the other side. Similarly, Dababat and Sikora (2007) showed induced systemic resistance in tomato plants against *M. incognita* when treated with *F. oxysporum* Fo162 in a split-root experiment.

So far, there have been relatively few studies on the biochemical nature of the induced systemic resistance mechanism. In a study conducted by Jagdale et al., 2009, entomopathogenic nematodes and their symbiotic bacteria were reported to induce systemic resistance to plants via systemically activating the production of key defense enzymes: P-peroxidase, G-peroxidase and higher catalyse activities. Moreover, Selim (unpublished data, 2010) has recently demonstrated that the ISR involves the release of chemical compounds in the Responder root exudates that affect nematode-host recognition behavior.

The present study clearly demonstrated that Fe14 stimulates ISR which is involved in setting off additional mechanisms responsible for its biocontrol activity in rice.

4.3 Repellent effect of root exudates

The reduction in migration of *M. graminicola* towards the fungi-treated roots compared with the non-treated roots in the present study demonstrated the influence of changes in the root exudates on the host finding ability of the root-knot nematode.

It has been well known that nematodes are attracted to plant roots (Prot, 1980; Prot and Van Gundy, 1981) and root exudates (Riddle and Bird, 1985; Viglierchio, 1961). Nematode orientation behavior can be characterized as taxis (directed movement towards the stimulus source) or kinesis (change of rate of movement in relation to stimulus intensity) (Perry, 1996). The stimulatory effect of root exudates on nematode host finding and egg hatching is well documented (Jones, 1960; Klinger, 1965; Webster, 1969; Edmunds and Mai 1976; Prot, 1980; Nordmeyer and Sikora, 1982; Pline and Dusenbery, 1987; Dababat and Sikora, 2007). Webster (1969) demonstrated that egg

hatching of cyst (*Heterodera* spp.) and gall forming nematodes (*Meloidogyne* spp.) in the soil was stimulated by exudates of some plants. Furthermore, plant parasitic nematodes are capable of directed orientation, ie klinotaxis, towards a plant stimulus (Klingler, 1965; Nordmeyer and Sikora, 1983; Pline and Dusenbery, 1987) and they move towards concentration gradients (Prot, 1980). The attraction of nematodes to a redox potential on the root surface has been one of several theories explaining the host finding ability of nematodes. However, the distance of this redox potential that could be recognized by a nematode is limited to a distance of 1 mm (Klinger, 1965) with a power of 20-30 mV/mm (Jones, 1960).

There have been relatively few experiments performed on the behavior of plant-parasitic nematodes in response to their host root exudates. Grundler et al. (1991) demonstrated the aggregation and pre-infectional exploratory behaviour of *H. schachtii* J2 in response to mustard root exudates under *in vitro* conditions. They did not observe any nematode orientation and assumed that the J2 reached the attractant source by random movement. In contrast, Clemens et al. (1994) observed that higher concentrations of root exudate attractants induced *H. schachtii* orientation. Their results supported the occurrence of two behavioral searching states, stimulated and unstimulated that are due to external (e.g. chemicals, nutrient medium composition, temperature, light, etc) or internal (e.g. physiological stage, age, hunger, etc) factors in the host-parasite interaction. In addition, only a few host or non-host specific compounds mediating the attraction of host to nematode are known. For instance, maize roots exude cyclic hydroxamic acids, one of which (2,-4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) attracts *Pratylenchus zeae* at concentrations in host exudates. Such compounds may be involved when endophytes are present (Chitwood, 2002).

Amino acids and carbon dioxide are the most attractive chemicals to nematodes in the root exudates (Bird, 1959; Klingler, 1963; Pline and Dusenbery, 1987; Prot, 1980). Diez and Dusenbury (1989) suggested that carbon dioxide is the principle means by which nematodes locate host roots. Both glutamic acid and aspartic acid tended to repel *Ditylenchus dipsaci* at concentrations of 1:1000 and attract them at 1:100 000 (Jones, 1960). Edmunds and Mai (1966) showed that *Pratylenchus penetrans* was preferentially

attracted to the high carbon dioxide emission of fungal infected roots. Similarly, Pline and Dusenbery (1987) illustrated that a sudden increase in CO_2 concentration caused an increase in locomotion of *M. incognita* and a decrease in the frequency of changes of direction. The threshold was about 0.01 % vol CO_2/vol gas when the baseline concentration was very low and 0.05% CO_2 when the baseline concentration was 1% CO_2. Furthermore, Nordmeyer and Sikora (1983) demonstrated that penetration of *Heterodera daverti* into subterranean clover roots was increased by a short exposure of the roots to a culture filtrate of *Fusarium avenaceum* because the culture filtrate increased the ion efflux of the clover roots by softening the root tissue and thereby enabled the nematode to penetrate more easily.

Root exudates can also have repellent effects to plant parasitic nematodes. Diez and Dusenbury (1989) reported that the root exudates from tomato plants having a repellent effect to *M. incognita* contained chemicals that showed up as different peaks in HPLC analysis. It was shown that root exudates appeared to contain only repellent activity because the other volatile stimuli were not captured by the described assay. Similarly, repellent effect of root exudates from tomato plants treated with non-pathogenic endophytic *F. oxysporum* strain 162 to *M. incognita* was reported by Dababat and Sikora (2007). However, inoculation of endophytic fungi to plants did not always result in repellent effects toward plant parasitic nematodes. For example, Viljoen et al. (2006) reported that the inoculation of the endophyte *F. oxysporum* to tissue culture banana plants did not alter host preference of *Radopholus similis*. These authors suggested that the reduction of nematode infection due to fungal treatment may occur at later stages, the so-called post infection stage.

The present study demonstrated that root exudates from Fe14 inoculated plants repelled or lacked of attractants to the root-knot nematode, indicating that Fe14 root exudates might contain chemicals, hormone or enzymes which are physio-chemically different from control plant exudates. This finding was similar to the results obtained by Padgham and Sikora (2007) on rice using an endophytic bacterium *Bacillus megaterium*. They demonstrated that rice plants treated with the bacterium negatively affected the host finding ability of *M. graminicola*.

4.4 Nematode development and reproduction

The endophyte *F. moniliforme* isolate Fe14 influenced *M. graminicola* development causing the production of a higher number of males in the Fe14 treated plants. The sex ratio in nematodes can vary and is greatly influenced by both genetic and environmental factors of which the latter is more profound and of interest for nematode control (Johnson and Viglierchio, 1969; Triantaphyllou, 1973; Papadopoulou and Triantaphyllou, 1982; Grundler et al., 1991). In general, the non-genetic factors affecting sex ratio are initial inoculum (Evans and Fox, 1977), host plant growth stage and resistance level (Trudgill et al., 1967, Berge et al., 1974, Lelivelt and Hoogendoorn, 1993; Sijmons, 1993; Anwar and McKenry (2000)), nutrient availability and the quality of giant cells (Ferris et al., 1984; Grundler et al., 1991; Sijmons, 1993)

Meloidogyne species are able to reproduce by mitotic parthenogenesis and when the environment is favourable, most juveniles will develop into females (Trudgill, 1997). It is necessary to consider root-knot nematode behavior during the early stages of the infection process in order to understand the interrelation between the host and nematode development and reproduction. First of all, the second stage juveniles are attracted to the zone of elongation, where they penetrate the root and then migrate intercellularly. This process involves both mechanical force and enzymatic secretions from the nematode. Once the nematode reaches the zone of differentiation, procambial cells adjacent to the head of the nematode develop into "giant cells" in response to signals from the nematode. These large, multinucleate, metabolically active cells serve as a permanent source of nutrients for the endoparasite and the nematode now is regarded as a nutrient sink (Williamson and Hussey, 1996). The quality of giant cells is an important factor determining male to female ratio (Ferris et al., 1984). Grundler et al. (1991) showed that the sucrose and amino acid contents of the synctia affected cyst nematode *H. schachtii* development and the male to female ratio. When the environment is favourable, most juveniles developed into females.

Furthermore, the resistance level of the host is also an important factor determining male to female ratio. High resistance normally leads to low number of females indicating poor development status. Lelivelt and Hoogendoorn (1993) demonstrated that

populations of *Heterodera schachtii* in resistant varieties of sugar beet consisted of more males than females. Rice et al. (1987) conducted an experiment to investigate the effect of the resistant gene H7 in potato against the cyst nematode *Globodera rostochiensis* and reported that the feeding site became necrosis and eventually collapsed. Ten days after penetration, the majority of nematodes remained in the later second or early third juvenile stage and the few nematodes that developed on H7 potato plants were mostly males, a sign of poor nutrition for the nematode.

In the present experiment, the significantly higher number of males in Fe14 treated plant might be attributed the poor quality of giant cells or the systemic resistance induced by the colonization of Fe14 in the endorhiza. This finding is particularly important because males are a non infective reproductive stage and thus higher numbers of males or lower numbers of females will lead to lower nematode populations and less damage to the root.

Moreover, the percentage of juveniles in plants treated by *F. moniliforme* Fe14 in the present study was higher than in control plants. This suggests a suppressive interaction between the fungal endophyte and the plant which prolongs the time needed to complete the life cycle of *M. graminicola*. Similar effects of the endophytic *F. oxysporum* on development of the burrowing nematode *R. similis* on banana and of the root-knot nematode *M. incognita* on tomato were reported by Niere (2001) and Vu (2005) and Dababat (2007) respectively.

Female fecundity or the egg production rate is an important determinant of nematode reproduction and population development and is influenced by environmental conditions and host status. In the present study, Fe14 not only reduced juvenile penetration but also altered the reproduction capacity of the females. The lower number of eggs per female in Fe14 treated plants suggested a post infection influence of the fungus on the nematode inside the host plant. Ferris et al. (1984) and Anwar and McKenry (2000) demonstrated that egg production rates of *M. arenaria* differed amongst grape varieties indicating a direct influence of the host physiology on female fecundity.

The effect of the biocontrol agent *Pochonia chlamydosporia* (syn. *Verticillium chlamyclosporium*) on the egg laying capacity of female nematodes was demonstrated by Kerry (1990). Number of eggs per beet cyst nematode was 90% lower when plants were treated with *P. chlamydosporia* compared to that of the control. In comparison, the mutualistic *F. oxysporum* Fo162 displayed no effect on female fecundity of *M. incognita* on tomato (Dababat, 2007). However, there are other mechanisms that the fungal antagonists can use to influence female fecundity. For examples, *P. chlamydosporia* affects *H. schachtii* by reducing the numbers or sizes of females or causing high infection rate of eggs (Kerry et al., 1982).

4.5 Biological control activity under oxic and anoxic soil conditions

The mutualistic endophytic *F. moniliforme* isolate Fe14 was able to reduce *M. graminicola* infection effectively under both soil water environments. Many studies on the biological control of a wide range of plant parasitic nematodes have been conducted under aerobic conditions (see previous sections of this chapter for more discussions). However, no research exists under anaerobic conditions. Therefore, this is the first report of a micro-organism that exhibits high levels of biocontrol activity against a plant parasitic nematode under anoxic soil environments. The findings are very important because it demonstrates the potential of using biological control technology against *M. graminicola* or other parasites such as *Hirschmaniella* species that are highly adapted to anoxic conditions under which other conventional control measures such as chemicals do not work or cannot be applied.

The infestation, development and reproduction of *M. graminicola* under different soil water regimes have been well studied (Bridge and Page, 1982; Prot and Matias, 1995; Soriano et al., 2000; Padgham et al., 2003). In general, the total nematode number is greater but the root galling index is lower under the flooded than non flooded conditions (Prot and Matias, 1995; Tandigan et al., 1996; Soriano et al., 2000). This fact can be explained by greater food supply due to better plant growth under flooding conditions, the tolerance of rice cultivars to the nematode or the limited spread of nematode infestation within the root system resulting in greater development of the nematode in the endorhiza. In comparison, the nematode is adversely affected in roots subjected to

long flooding periods over 5 months due to the depletion of root oxygen supply in the old roots (Bridge and Page, 1982) and this probably explains the smaller number of nematode under flooding conditions in our experiment.

5. Conclusion

The mechanisms responsible for the biocontrol activity of the non-pathogenic endophyte *F. moniliforme* Fe14 were investigated through different types of experiments. From the experimental results it can be concluded that:

1) Fe14 reduced significantly nematode penetration of rice roots under oxic conditions

2) Induced systemic resistance was clearly demonstrated when Fe14 colonized rice in split-root study

3) Root exudates from Fe14 treated plants have a repellent activity against *M. graminicola* in the absence of rice indicating changes in the root exudates or a release of toxic substances produced or induced by Fe14

4) Fe14 altered *M. graminicola* development and reproduction represented by a higher number of males or lower number of females as well as less eggs per female in the Fe14 treated plants and therefore directly influenced nematode parasitism

5) Fe14 exhibited high levels of biocontrol under anoxic soil conditions

CHAPTER 5: Importance of inoculation time and method of application

1. Introduction

Biocontrol efficacy is usually inconsistent due to various abiotic and biotic factors and the complex interactions between these factors after application. Important factors affecting biocontrol are inoculum production and the form of application. It has been demonstrated that biocontrol of various plant parasitic nematodes depends on the inoculum density, time and forms of application (Vu, 2005; Dababat and Sikora, 2007). Most researchers used fungal and bacterial inoculum in the range of 10^5-10^8 cfu/ g soil to control nematodes (Vu, 2005; Padgham and Sikora, 2007; Dababat, 2007; Mendoza, 2008). In addition, the methods of application also vary considerably. Some researchers applied fungi or bacteria by soil drenching or soil incorporation (Hallmann and Sikora, 1994; Vu, 2005; Dababat, 2007; Mendoza, 2008) while others coated the seeds with biocontrol agents (Padgham and Sikora, 2007).

Time of antagonist introduction is an important factor in governing its efficacy. Protection of plant at the seedling stage from nematode infection is very important for subsequent production and yield. The seedling is the most susceptible stage to pest and disease infection because it is not fully developed and therefore unable to confront adverse biotic and abiotic factors effectively. Thus, many agrochemicals or biological control agents are developed to protect plants in this early stage.

Cabanillas and Barker (1989) reported that *Paecilomyces lilacinus* was more effective in protecting tomato against *M. incognita* when it was delivered before transplanting or at transplanting than after plants were infected by nematodes. Similar results were obtained when tomato or banana plants were treated with the mutualistic endophyte *Fusarium oxysporum* Fo162 at transplanting (Vu, 2005, Mendoza et al., 2006; Dababat and Sikora, 2007). In comparison, post-planting application of biocontrol agents, especially in the case of endophytes does not always lead to high levels of biocontrol since the establishment of a biocontrol agent in the endorhiza or the rhizosphere is a

prerequisite for the control of endoparasitic nematodes (Vu, 2005; Mendoza et al., 2006; Dababat and Sikora, 2007).

However, not all biocontrol agents are able to protect seedlings due to lack of rhizosphere competency. Therefore, finding the right organisms, optimal application time and the form of application is essential to improve biocontrol efficacy. For example, with many endophytes, a certain period is required for the control agent to establish and interact with the host plants so as to express effective control efficacy (Vu, 2005; Dababat and Sikora, 2007). Understanding the biochemical and ecological interactions is the key to successful biocontrol.

Adequate application form is an essential part of biocontrol because it influences survival and colonization of the biocontrol agent. Antagonists including fungi and bacteria can be applied in the form of spore suspensions or living cells with or without metabolites. In the case of endophytes, the biocontrol agents should be applied in advance before exposing the plants to nematode because they need time to establish and reproduce inside the endorhiza of the host plants. The objectives of this chapter were to:

1) Investigate the ability of fungal antagonists to protect plants in the seedling stages

2) Determine the optimal inoculation time

3) Compare soil drenching and seed coating methods for effectiveness

2. Experimental design

General materials and methods are described in chapter 2. Only specific changes in methodology are given in this chapter.

2.1 Fungal and nematode inoculation at sowing

Three endophytic fungal isolates, *F. moniliforme* Fe1 and Fe14, *F. oxysporum* Fo162 and two rhizosphere isolates *Fusarium* F28 and *Trichoderma* T30 were tested for their ability to provide early protection of rice seedlings against *M. graminicola* infection. Seed treatment technology was applied in this study. Rice seeds were first surface sterilized and pre-germinated as described in chapter 2. The fungal biomass of each isolate was then mixed with 2% methyl cellulose and then coated to the germinating seeds for 2 hours (See chapter 2). Seeds coated with methyl cellulose alone served as the control. The coated seeds were then sown in experimental pots measuring 7x7x8 cm filled with 300 g of autoclaved sandy soil. After treatment, two seeds of each treatment were examined for colony forming unit (cfu) attached by shaking them vigorously in 10ml sterilized tap water. The cfu was determined using the Jetset spiral plater (See chapter 2). The cfu per coated seed was approximately 10^6 for each fungal isolate. Immediately after sowing, fresh second stage juvenile (J2) of *M. graminicola* were drenched onto the soil at a rate of 250 J2/pot (approx. 1 J2/ g soil). The pots were kept moist to ensure J2 migration and plant growth. Three weeks after sowing, the rice seedlings were uprooted and washed carefully under tap water to remove adhering soil. Fresh root weight was recorded and the number of galls was counted using a magnifying glass.

The experiment was repeated with only 2 isolates Fe14 and T30 based on the effects obtained in the first test. The treatments were conducted in the same manner.

2.2 Long term biocontrol activity

The endophytic *Fusarium moniliforme* isolate Fe14 was previously studied for its biocontrol activity when it was inoculated to the rice seeds using seed treatment technique 4 weeks after sowing. The current experiment was conducted to examine

whether the endophyte maintained its biocontrol activity against *M. graminicola* over time.

The fungal biomass of the isolate Fe14 was coated to the pre-germinating rice seeds as previously described in the section 2.1 of this chapter. The second fungal inoculation was made three weeks later by soil drenching. Ten weeks after the second fungal inoculation, 1000 J2 were inoculated into the rhizosphere of a rice plant through 3 holes made with a plastic rod around the stem base. The experiment was terminated two weeks later. All roots were washed and then stained with 1% Fuchsine acid. The penetration rate of *M. graminicola* was determined as previously described in chapter 4, section 2.1.

2.3 Drenching versus seed treatment

Control efficacy of Fe14 was evaluated using different forms of fungal inoculation: seed coating, soil drenching or a combination of both. The methods of seed coating or soil drenching have been described in chapter 2 and also in other chapters. The experiment was designed with 5 treatments, namely: coating (Cg), drenching (D), coating and soil drenching (Cg+D), drenching twice (2xD) and the control treatment (C). For the combination of seed coating and soil drenching, the fungal biomass was firstly coated to the pre-germinating seeds and three weeks later, additional fungal spores were drenched to the experimental pots. For the double soil drenching treatment (2xD), the first drench of fungal spores was made at sowing and then repeated three weeks later at a dose of $5x10^6$ spores/seedlings each time. Four weeks after sowing, all rice seedlings were inoculated with *M. graminicola* at a density of 500 J2/ plant. The experiment was harvested 6 weeks after nematode inoculation. Roots were washed and stained with 1% Fuchsine acid and the number of nematodes inside the root was counted under binoculars.

3. Results

3.1 Fungal and nematode inoculation at sowing

Inoculation of rice seeds at sowing with the endophytic isolates Fo162, Fe1 and Fe14 provided no protection from root-knot nematode infestation. The rhizosphere isolates F28 and *Trichoderma* T30 reduced galling severity by 7% and 19% respectively. However, there was no significant difference between treatments (Fig. 5.1).

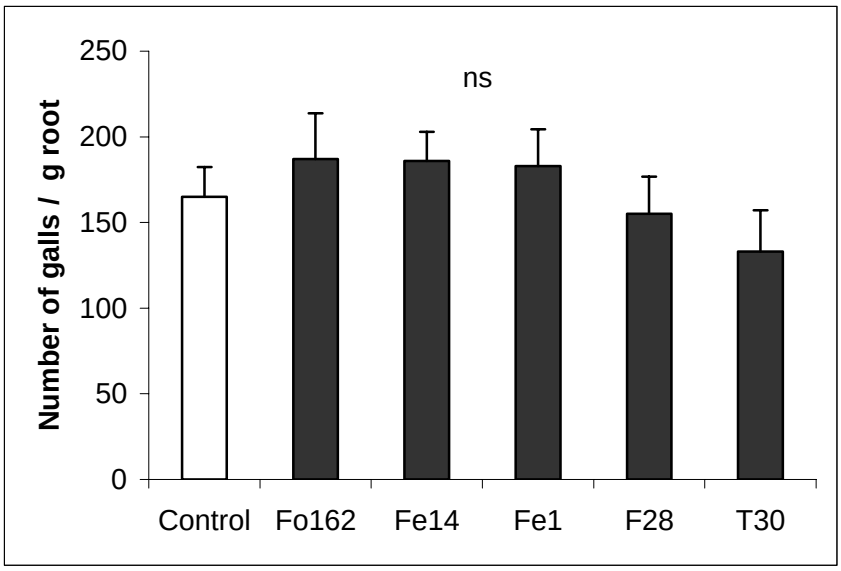

Figure 5.1: Effect of the fungal endophytes *Fusarium oxysporum* Fo162, *Fusarium moniliforme* Fe1 and Fe14 and rhizosphere antagonists *Fusarium* F28 and *Trichoderma* T30 on galling severity caused by *Meloidogyne graminicola* in rice root when the nematode was applied at sowing. Bars represented standard errors of the mean, ns: not significantly different according to the LSD test ($P \leq 0.05$; n=7).

Moreover, growth of rice seedling was slightly reduced in treatments with both nematode and fungal inoculations. Fungal inoculation alone did not influence the rice shoot weight and root weight significantly. The height of rice plants was not affected by fungal or nematode inoculation (Table 5.1)

Table 5.1. Effect of seed treatments with the endophytes *Fusarium oxysporum* Fo162 and *F. moniliforme* isolates Fe1 and Fe14 and the rhizosphere antagonists *Trichoderma* T30 and *Fusarium* F28, and nematode inoculation at sowing on growth of rice 3 weeks after sowing.

Treatment	With *M. graminicola*			Without *M. graminicola*		
	Root weight (g)	Shoot weight (g)	Shoot height (cm)	Root weight (g)	Shoot weight (g)	Shoot height (cm)
Control	0.26 a	0.17 ab	20.5 ab	0.22 a	0.17	20 a
T30	0.27 a	0.19 a	21.5 a	0.21 ab	0.18	21 a
F28	0.22 ab	0.16 ab	20.0 ab	0.24 a	0.16	20 a
Fo162	0.18 bc	0.14 bc	18.0 bc	0.18 b	0.16	20 a
Fe14	0.15 c	0.10 ce	16.0 c	0.20 ab	0.16	20 a
Fe1	0.14 c	0.09 e	16.0 c	0.17 b	0.15	18 b
P -value	0.000	0.000	0.005	0.038	ns	0.001

Means in the same column followed by the same letters are not significantly different according to the LSD test ($P \leq 0.05$, $n = 7$). ns: not significantly different.

In the second seed treatment experiment, only Fe14 and T30 were selected for the test on their ability to provide early protection for the rice plant. Seed treatment of Fe14 did not lead to a decrease in galling whereas the *Trichoderma* isolate T30 reduced galling severity significantly when compared with the control and Fe14 treatments (Fig. 5.2).

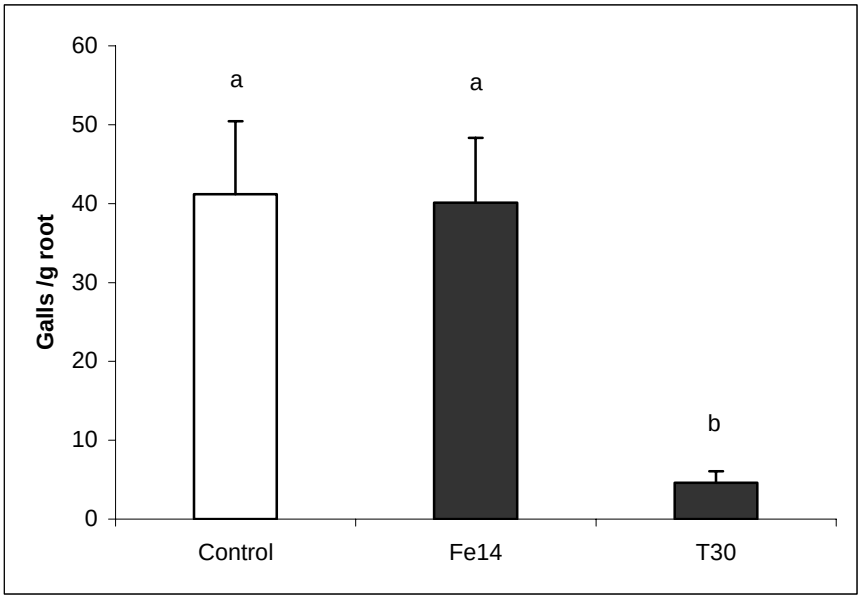

Figure 5.2: Effect of the endophyte *Fusarium moniliforme* Fe14 and the rhizosphere *Trichoderma* T30 applied as seed treatment at sowing on *Meloidogyne graminicola* infestation in rice. Means with the same letters are not significantly different based on LSD test (P≤0.05; n=7). Bars represented standard errors of the mean.

Seed treatment with Fe14 or T30 slightly increased root weight whereas the shoot height was similar amongst all treatments. The shoot weight of rice seedlings treated with T30 was slightly higher compared to that of the control and Fe14 treatments (Table 5.2).

Table 5.2: Effect of seed treatment with the endophyte *Fusarium moniliforme* Fe14 and the rhizosphere *Trichoderma* T30 on growth of rice 3 weeks after sowing when *Meloidogyne graminicola* was inoculated at sowing.

Treatment	Root weight (g)	Shoot weight (g)	Shoot height (cm)
Control	0.5	0.30	20.6
T30	0.49	0.39	22.6
Fe14	0.39	0.30	22.8
P-value	ns	ns	ns

(ns: not significantly different based on LSD test (P≤0.05; n=7))

3.2 Long term biocontrol activity

Nematode penetration was reduced approximately 55% when the nematode was introduced to the rice plants treated with the mutualistic fungus *F. moniliforme* Fe14 ten weeks earlier.

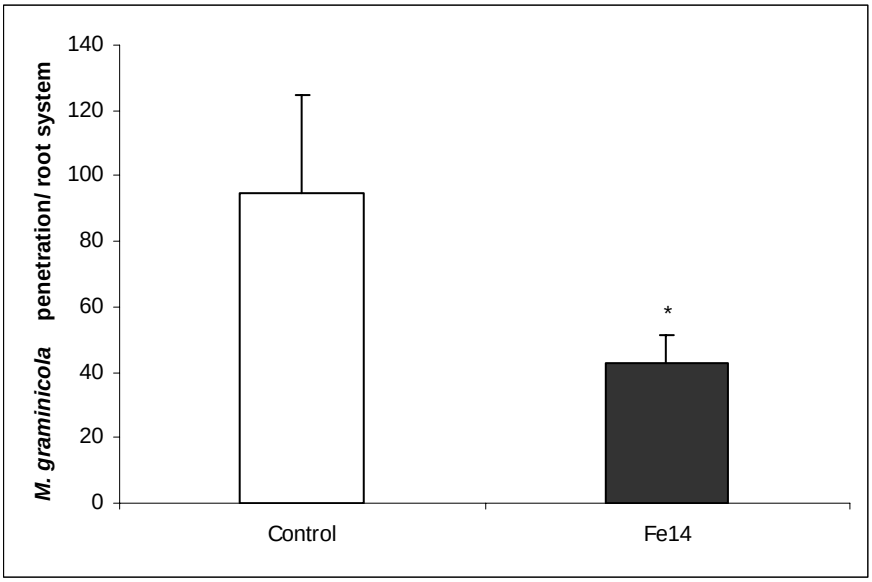

Figure 5.3: Effect of fungal isolate *Fusarium moniliforme* Fe14 inoculated to rice roots for 10 weeks before *Meloidogyne graminicola* was introduced. * = significantly different based on the T-test ($P \leq 0.05$; n=7). Bars represented standard errors of the mean.

3.3 Drenching versus seed treatment

The form of inoculation affected the level of biocontrol toward the rice root-knot nematode. In general, all methods of application reduced nematode infestation significantly by 19-35% when compared to the control treatment. Seed coating or soil drenching alone protected the plants from infection better than when the fungus was applied to rice by a combination of seed coating and soil drenching (Figure 5.4).

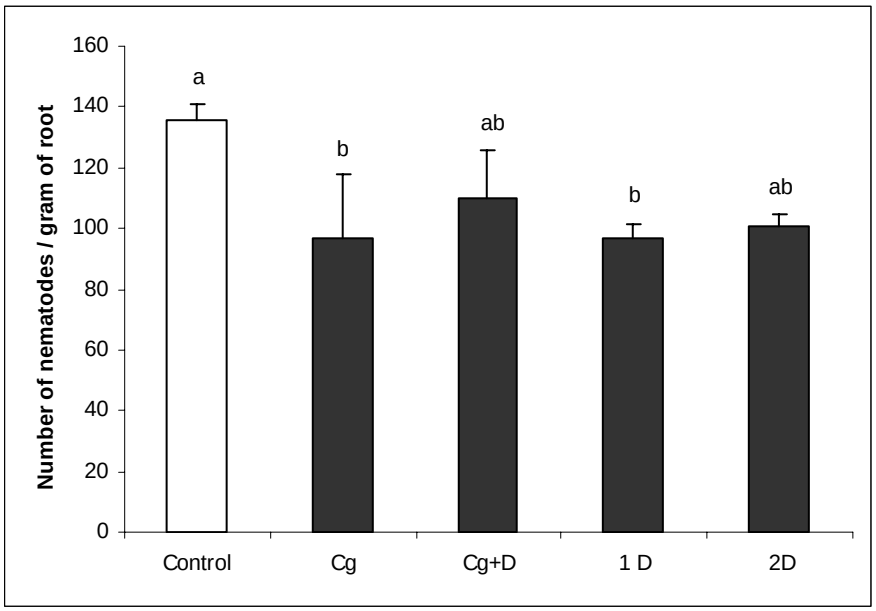

Figure 5.4: Effect of application form of the fungal isolate *Fusarium moniliforme* Fe14 as a seed coating (Fe14Cg), coating and soil drenching (Fe14Cg+D), drenching (Fe14 D), drenching twice (Fe14 2D) and the control (C), on *Meloidogyne graminicola* population densities in rice root. Means with the same letters are not significantly different based on LSD test ($P \leq 0.05$; n=7). Bars represented standard errors of the mean.

4. Discussion

4.1 Fungal and nematode inoculation at sowing

All of the fungal isolates, except the *Trichoderma* T30 did not protect rice seedling from *M. graminicola* infestation when introduced simultaneously at sowing. The slightly higher gall number in the endophytic fungi treated plant was probably due to the low level of the endophytes in the endorhiza when the nematode was penetrating. Inoculation of rice with the fungal isolates F28 and T30 slightly reduced the nematode infestation to the rice root. This could be explained by the fact that these two isolates were rhizosphere fungi. Their mode of action is different than the endophytic fungi which need time to colonize the root for biocontrol activity. The isolates F28 and T30 might produce antimicrobial substances that distracted the host finding of the nematode in a very short time or are pathogenic to the J2. The endophytic fungi Fe1, Fe4 and Fo162 did not reduce nematode infestation. The results of this experiment are in agreement with that of Vu (2005) and Dababat Sikora (2007). They demonstrated that the endophytic fungus *F. oxysporum* Fo162 expressed no immediate protection to the plant when exposed to nematode simultaneously at inoculation time. As endophytes, the fungi need time to establish inside the root tissue and interact with the rice roots before they can express their biocontrol activity.

Some species of *Trichoderma* have been demonstrated biocontrol activity toward root-knot nematodes (Windham et al., 1989; Meyer et al., 2001; Sharon et al., 2001). The lower number galls formed in plants treated with T30 could be explained by the direct effect of toxic substances secreted by the isolate or the disturbance of root exudates caused by this fungus (Lorito et al., 1996).

4.2 Long term biocontrol activity

The mutualistic fungus *F. moniliforme* Fe14 showed long term biocontrol activity against *M. graminicola* in rice. A reduction in galling severity was still observed when the nematode was introduced to rice plants pre-inoculated with Fe14 ten weeks earlier. Similar results were reported by Vu (2005) when she investigated the biocontrol activity

of 4 endophytic *F. oxysporum* isolates A1, Fo162, W5W2 and H-20 against the burrowing nematode *R. similis* on banana. High biocontrol activity was still obtained when the fungi were pre-incubated 14 weeks in banana. Niere (2001) also observed high biocontrol activity of the endophytes *F. oxysporum* over a 5 months period. The results of the present study demonstrated that the mutualistic fungus Fe14 grew and developed well in the endorhiza and retained high levels of antagonism toward *M. graminicola* over time.

4.3 Drenching versus seed treatment

Forms of application are important in biological control. Depending on the antagonists, such as fungi, bacteria or the place where they reside, the forms of application may vary accordingly. In the present study, it was shown that application techniques either by seed coating and/ or soil drenching significantly influenced biocontrol level. The seed coating, combined coating and soil drenching or drenching twice gave similar levels of biocontrol.

For biocontrol technology, seed treatment is regarded as the most economical method for several reasons. First of all, it provides early protection to the seeds before plants are actually exposed to various biotic and abiotic stresses in the field. Secondly, seed treatment requires small amounts of inoculum and thereby reduces the overall cost of application (Harman, 1991; Sikora et al., 2003; Elzein et al., 2006). In the present experiment, it was clearly shown that seed treatment using the endophytic mutualistic Fe14 worked well in biocontrol of the rice root-knot nematode. This finding is important because seed treatment allows application of biocontrol agents on a large scale to seed with relatively low application cost.

Similarly, soil drenching is also a common method of biocontrol application in intensive production systems such as in the greenhouse or nursery because of high labor requirement. However, the efficacy is also high with soil drenching method. Studies conducted on the soil drenching method using the endophytic *F. oxysporum* Fo162 showed that the technique was adequate to give high level of biocontrol against *Radopholus similis* in banana or against *M. incognita* in tomato if the endophyte has

time to establish before being exposed to the nematode in the field (Vu, 2005; Dababat and Sikora, 2007).

Multiple applications of Fe14, either combining seed coating and soil drenching or drenching twice did not significantly increase the overall level of nematode control over that obtained with a single inoculation at sowing. The results demonstrated that the biocontrol activity of endophyte only obtained when it has up to 4 weeks time to colonize the roots before nematode inoculation. Similar results were reported by Dababat and Sikora (2007) when they studied the effect of single or dual applications of the mutualistic endophytic *F. oxysporum* Fo162 for biocontrol of *M. incognita* on tomato. They demonstrated that single application of the antagonist at sowing is adequate to obtain high level of biocontrol. In comparison, Mendoza and Sikora (2009) suggested a dual application of the egg pathogen *Paecilomyces lilacinus* PL 251 with an endophyte *F. oxysporum* Fo162 was necessary for effective biocontrol of the burrowing nematode *R. similis* on banana. The egg pathogen reduces nematode infection potential in the soil and gives the endophyte time to establish. The effect of single or multiple inoculations on biocontrol efficacy is probably dependent on the modes of action of the antagonists. For instance, Fo162, being an endophyte, requires a period of time to establish and express its biocontrol activity inside the host plant whereas PL 251 must be present in the rhizosphere with a substantial amount in order to infect eggs. Therefore multiple applications can be important if used strategically. In the present study, dual inoculation of Fe14, which did not lead to higher level of biocontrol, also adds to overall costs of the treatment and therefore is not recommended. The results suggest that seed treatment with fungal endophyte would be important for rice sown in seed beds before going to the field for transplanting. This would give the endophyte time to establish in the endorhiza before it can express its biocontrol activity against the nematode.

5. Conclusion

The biocontrol efficacy of the endophyte *F. moniliforme* Fe14 against *M. graminicola* in rice was evaluated under different inoculation time and methods. From the experimental results the following conclusions can be made:

1) Fe14 requires an establishment period in the endorhiza in order to achieve high levels of biocontrol against the root-knot nematode

2) Fe14 retained high levels of biocontrol over time

3) Both seed treatment and soil drenching technique are effective inoculation methods to obtain colonization although seed treatment is recommended due to lower labour and application cost

4) Single inoculation of Fe14 is adequate to achieve high levels of biocontrol over time

CHAPTER 6: Influence of multiple combinations of microbial antagonists on biocontrol activity

1. Introduction

Numerous microbes are antagonistic to plant pests and pathogens and some of these micro-organisms have been demonstrated to suppress plant parasitic nematode populations (Sikora 1992; Kerry, 2000; Meyer and Roberts, 2002; Sikora et al., 2007). Despite a substantial amount of research on the antagonistic potential of microbes, the application of biological control agents in the field is still limited. Reasons for this drawback include inconsistent performance, complicated interactions with other organisms in the endo- and rhizosphere and slower action of the control agents compared to pesticides (Meyer and Roberts, 2002). In addition, mass production, formulation, registration, marketing, delivery and application techniques can complicate commercialization of microbial based biocontrol agents (Kerry, 2000; Meyer and Roberts, 2002). Only a few products are available for nematode management on the market including *Burkhoderia cepacia, Trichoderma virens*, *Paecilomyces lilacinus* strain 251, *Bacilus firmus, Pasteria penetrans and Pochonia chlamydosporia* (syn. *Verticilium chlamydosporum*) (Meyer and Roberts, 2002; Mendoza, 2008). To overcome inconsistent performance of biocontrol agents and to improve biocontrol efficacy, combinations of more than one microbe are recommended (Mao et al., 1998; Kerry, 2000; Meyer et al., 2001; Mendoza and Sikora, 2009; Sikora et al., 2010).

Soil ecosystems harbor millions of living organisms from microscopic to megascopic scales. The activity of one organism is usually affected by others besides the common influence of abiotic factors. It was discovered that nematode suppressive soils usually contain a wide range of natural enemies that attack their nematode host at different stages in the life cycle (Kerry 1990; zum Felde et al., 2006). Each organism may kill relatively few nematodes but the combined effects of several antagonists may suppress nematode population. Therefore, many nematologists consider that combination application of different antagonists with different modes of action can increase effectiveness of integrated nematode management programs (Kerry, 1990, Sikora, 1992; Sikora et al., 2007).

The *in vitro* inhibition test is a common method to study antagonism between two or more organisms. Compatibility is observed when the growth of one isolate does not inhibit that of the other in the same culture medium. Inversely, when the growth of an organism slows down or is limited by the presence of the other, the two microorganisms are said to be incompatible. There are several ways of assessing the interaction, among those, the interaction category set by Stahl and Christensen (1992) has been widely accepted and has been used in this study. Neutral intermingling occurs when two microorganisms grow and develop normally in the dual culture. Both of them can overgrow each other in the Petri dish. In contrast, the interaction is the deadlock type when one of the two isolates inhibits the growth of each other whereby a distinct zone appears between the 2 tested organisms. Antibiotics or metabolites produced by one may inhibit the growth of the other. One isolate is said to replace the other when its mycelia partially or completely covers those of the other (Stahl and Christensen, 1992).

In an attempt to improve the stability, intensity and reliability of biocontrol, numerous authors have studied the effect of combining biocontrol agents on biocontrol of plant parasitic nematodes (reviewed by Meyer and Roberts, 2002). In many cases, the combinations led to an increase in biocontrol level (Guetsky et al. 2001, Guetsky et al. 2002, Meyer and Roberts 2002). Combinations of biocontrol agents against nematodes include the use of fungi with fungi (Sikora and Hoffmann-Hergarten, 1993; Khan et al. 1997, Duponnois et al. 1998, Hojat Jalali et al. 1998, Chen et al. 2000, Masadeh et al., 2004; zum Felde et al., 2006; Mendoza et al., 2006, Chaves et al., 2009) and fungi with bacteria (Maheswari and Mani 1988; de Leij et al. 1992; Siddiqui and Mahmood 1993; Esnard et al., 1998; Perveen et al. 1998; Chen et al. 2000, Mendoza and Sikora 2009; Chaves et al., 2009). Most combinations involved two organisms, but few used combinations of three or more organisms (Esnard et al. 1998; Chen et al. 2000; zum Felde et al., 2006; Mendoza and Sikora, 2009). However, combinations are not always synergistic or beneficial, as antagonism can occur between biocontrol organisms, and lead to unchanged control levels (Zaki and Maqbool 1991, Viaene and Abawi 2000) or even to lower control (Esnard et al. 1998, Chen et al. 2000, Meyer et al., 2001, Masadeh et al., 2004), when compared to individual applications of biocontrol agents.

Although combinations using several biological control agents have been extensively studied in biological control of plant parasitic nematodes results are always unpredictable due to the complication of the behaviour and interaction of the antagonists with other organisms. The objectives of these studies were to determine the:

1) *In vitro* compatibility of *Fusarium moniliforme* Fe14, *Trichoderma* T30 and *Bacillus megaterium* Bm

2) Efficacy of simultaneuos applications of three biological control agents Fe14, T30 and Bm on *M. graminicola*

3) Biocontrol effect of sequential applications of Fe14, T30 and Bm on *M. graminicola*

2. Experimental design

2.1 *In vitro* compatibility of Fe14, T30 and Bm

Compatibility of pairs of the antagonists *in vitro* was tested using a dual culture technique. Cultures of test fungi were grown on 100% PDA medium for ten days whereas the bacterium was multiplied on TSA and then TSB for one day (See chapter 2).

The compatibility between Fe14 and T30 was determined by cutting a disc of each of the antagonistic colonies (6 mm Ø) using a sterilized plug and placing them 4 cm away from each other on a 100% PDA plate. To determine the compatibility between the bacterium *Bacillus megaterium* Bm with either one of the two fungi, the bacterial isolate was streaked across the middle of a 100% PDA plate using a sterile plastic loop in a cross form. Then 4 plugs of the same fungus were placed at the outer edge of each quadrant of the cross.

Control plates were inoculated with only one of the two fungi or the bacterium. All plates were incubated in darkness at 25^{o}C for 5 days. Compatibility was determined when colonies were close to merging. Radial growth was measured every 2 days until day 10 and compared with colony growth on the control plates. Morphological changes in the antagonists and the merging zones were examined under light microscopes. The interaction between fungus-fungus and bacterium-fungus was classified as: (1) neutral intermingling, (2) deadlock and (3) replacement (Stahl and Christensen, 1992). Each treatment was replicated 5 times and the experiment was conduced twice.

2.2 Multiple applications of antagonists with different modes of action at sowing

The influence of multiple applications of different biocontrol agents was tested in two sets of experiments. In the first experiment, the endophyte Fe14 was combined with the egg pathogen T30 for the biocontrol of the root-knot nematode. Rice seeds were sterilized and pre-germinated as previously described in chapter 2. At sowing, the soil of the planting hole was drenched with $5x10^7$ spores of either T30 or Fe14 or $2.5x10^7$ spores each of both T30 and F14. Pots treated with tap water served as controls. Fungal inoculation was repeated by another drench three weeks later. Four weeks after sowing,

2 ml of tap water containing 1000 J2 were inoculated to each seedling. The nematode inoculum was applied into 3 holes around the rhizosphere. The plants were water daily and fertilized weekly with the Yoshida solution. The experiment was terminated three weeks later. Rice roots were washed carefully and the fresh root weights were recorded. Nematode infection level was assessed by counting the number of galls in each root system.

In the second set of experiment, one more biocontrol agent, the endophytic bacterium Bm was applied together with Fe14 and T30 followed the same experimental procedure as in the first experiment. Spores of Fe14 and T30 were collected and concentrations were adjusted to 10^7 spores/ ml. Cells of Bm were also extracted and then also adjusted to a concentration of 10^7 cfu/ ml using ¼ strength Ringer solution. Rice seeds were sown in experimental pots containing 250 cm^3 of sterilized sandy soil. Each rice seed received 5 ml of one bacterial or fungal solution containing $5x10^7$ cfu or 2.5 ml of each antagonist in combined treatments (2x 2.5x 10^7 cfu) (See chapter 2 for more detail of the culturing and determination of cfu of fungal and bacterial solution). Three weeks later, a second fungal or bacterial inoculation was applied. Control plants received the same amount of tap water or quarter strength Ringer solution. One week after the second inoculation of the fungi or the bacterium, 1000 J2 of *M. graminicola* were inoculated to each rice plant. The experiment was harvested three weeks after nematode inoculation. All plant roots were washed carefully and fresh root weight was recorded. Level of nematode infection was determined by counting number of galls per gram of root. Each treatment was replicated 8 times and the experiment was conducted twice.

2.3 Sequential application of Fe14, T30 and Bm

In this study, Fe14, Bm and T30 were applied to rice seeds and seedlings in a staggered time manner. Previous experiments and citations in the literature showed that *Trichoderma* is an egg pathogen to plant parasitic nematodes, including *Meloidogyne* species. Therefore, in this experiment, spores of T30 were mixed with nematode eggs in sterilized sandy soil to simulate the egg-pathogenic conditions in the field.

Rice seeds were surface sterilized and then pre-germinated on wet filter paper for 3 days. The germinating seeds were sown into experimental pots and were immediately inoculated with Fe14 and/ or Bm at concentrations of $5x10^7$ spores or cfu per seed respectively. Rice seedlings in combined treatment received each time 2.5 ml of both fungal and bacterial suspensions of the same concentration. Additional fungal or bacterial inoculation was made 3 weeks later by soil drenching with $5x10^7$ cfu of each.

Two weeks after sowing (i.e. two weeks after inoculating the rice seeds with Fe14 or Bm), a concentration of 10^5 spores of *Trichoderma* T30 was incorporated simultaneously with 20 nematode eggs per gram of sterilized sandy soil. The soil was mixed well with small amount of water and added to experimental pots and left for two weeks in the greenhouse. These pots were maintained at the same moisture level to facilitate the fungal activity and the living of nematode.

Four weeks after sowing, all rice seedlings which were pre-inoculated twice with Fe14 or/and Bm and control plants were transplanted to the pots containing the mixture of T30, eggs and/or J2 of *M. graminicola*. The experiment consisted of a control and 7 treatments: Bm, T30, Fe14, Fe14+Bm, Fe14+T30, T30+Bm and Bm+Fe14+T30 (All). Galling severity was assessed three weeks after nematode inoculation. Each treatment was replicated 8 times and the experiment was conducted twice.

3. Results

3.1 *In vitro* compatibility of Fe14, T30 and Bm

Compatibility of the antagonists Fe14, T30 and Bm with each other was studied using a dual inoculation technique on PDA medium. In culture plates, growth of T30 was slightly faster than that of Fe14 and the interaction between two isolates was of the deadlock type (Fig 6.1). Observations under the light microscope revealed no morphological changes in the mycelia of either fungus. Deadlock or self-inhibition also occurred in plates inoculated with cultures of only Fe14. In comparison, neutral intermingling was observed in dual culture of T30+Bm and T30+T30. Mycelia of T30 completely overgrew lines of the bacterium Bm while colonies of Fe14 were separated by Bm lines in the same plate.

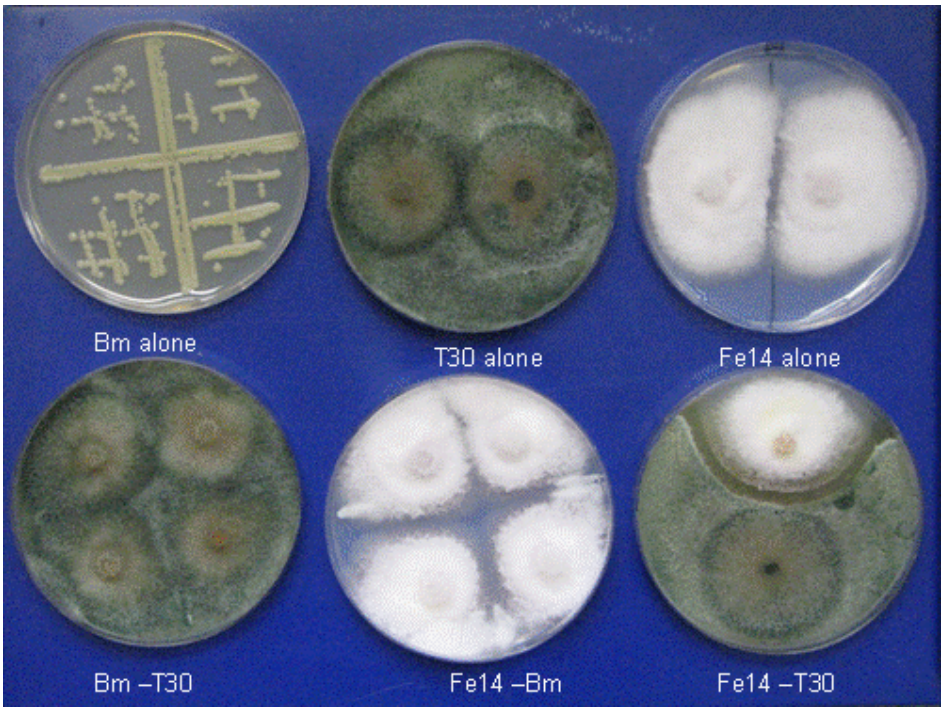

Figure 6.1: *In vitro* compatibility of *Fusarium moniliforme* (Fe14), *Trichoderma* (T30) and *Bacillus megaterium* (Bm) on Potato dextrose agar plates after incubation at 25^{o}C in darkness for 14 days.

In this experiment, the growth rate of the 2 fungal strains Fe14 and T30 was measured every 2 days until day 10. The average growth rate of Fe14 and T30 was 1.5 and 3 cm

respectively. The growth rate of Bm was slow and did not exceed 1 mm of the original streak on the culture medium (Data not shown). The slow growth rate of Fe14 in the presence of *Trichoderma* on the same plate indicated a slight inhibition effect of T30 over Fe14.

3.2 Multiple applications of antagonists with different modes of action at sowing

Multiple applications of the two isolates Fe14 and T30, when applied individually or in combination reduced the root-knot nematode galling significantly. Combination of Fe14 and T30 significantly lowered nematode galling 67% over that of the control. However, the level of biocontrol was not significantly different between the single or combined inoculations (Figure 6.2).

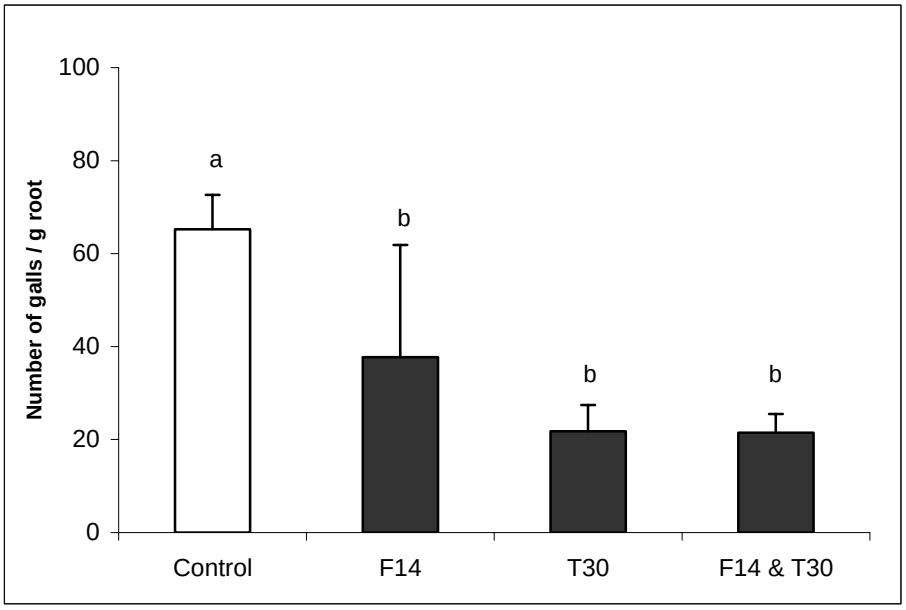

Figure 6.2: Effect of single or combined application of *Trichoderma* isolate T30 and the endophytic fungus *Fusarium moniliforme* Fe14 on the infection of *Meloidogyne graminicola* in rice. Vertical bars represent standard errors of the means. Columns having the same letters are not significantly different based on the LSD test ($p \leq 0.05$, n=7).

In the second experiment, all forms of inoculation reduced *M. graminicola* infestation. Applications of one or two antagonists (Bm, Fe14, T30, Fe14+T30; Bm+T30) reduced gall formation significantly by 20-38%. Combination of Fe14 and T30 lowered nematode infestation but the reduction was not significant compared to the control (Fig. 6.3).

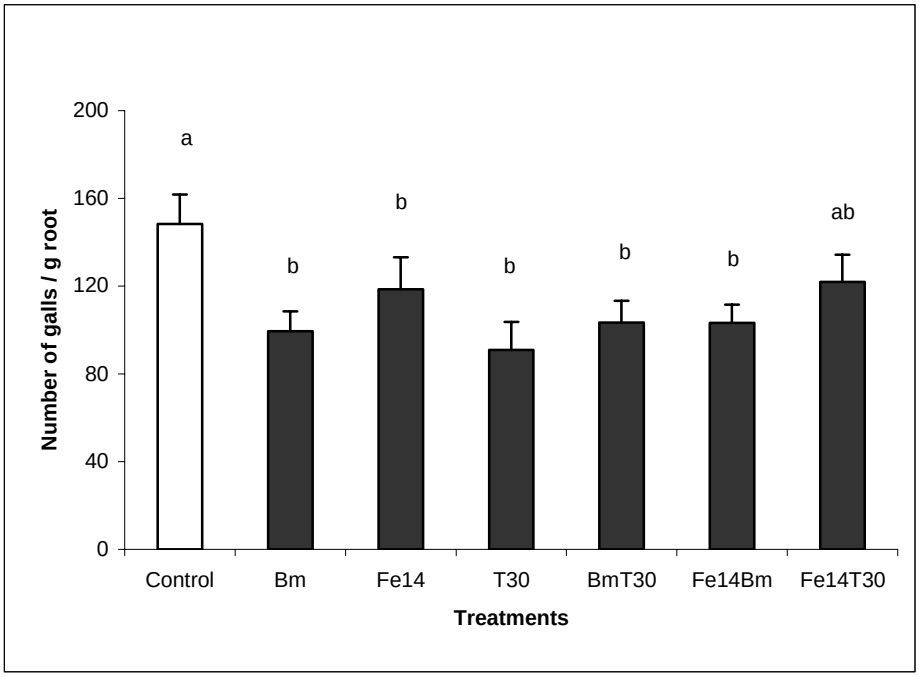

Figure 6.3: Effect of individual or multiple applications of *Fusarium moniliforme* (Fe14), *Trichoderma* (T30) and *Bacillus megaterium* (Bm) on *Meloidogyne graminicola* infestation in rice roots 7 weeks after sowing under greenhouse conditions. Vertical bars represent standard errors of the means. Columns having the same letters are not significantly different based on the LSD test ($p \leq 0.05$, n=16).

In general, the three biological control agents, when applied individually or in combination to rice plants did not affect growth of rice significantly. Root weight, shoot weight and shoot height however was improved in most treatments. Only the bacterium *B. megaterium* Bm increased the shoot weight of rice significantly over the control and Fe14 treated plants (Table 6.1).

Table 6.1: Effect of single or simultaneous application of *Bacillus megaterium* (Bm), *Fusarium moniliforme* (Fe14) and *Trichoderma* (T30) on growth of rice 7 weeks after planting under glasshouse conditions.

Treatments	Root weight (g)	Shoot weight (g)	Shoot height (cm)
Bm	1.04	0.99 b	29
Fe14	0.78	0.79 a	27
T30	0.92	0.89 ab	29
Bm+T30	0.89	0.84 ab	25
Fe14+Bm	0.90	0.91 ab	27
Fe14+T30	0.86	0.86 ab	26
Control	0.81	0.81 a	25
P value	ns	0.039	ns

Columns having the same letters in a column are not significantly different based on the LSD test ($p \leq 0.05$, n=16).

3.3 Sequential applications of Bm, Fe14 and T30

All fungal or bacterial treatments in experiments 1 and 2 reduced nematode infection. In the first experiment, all inoculation methods reduced the nematode infestation significantly by 25-78% compared to that of non-treated control (Table 6.2). Combination of Fe14 and T30 resulted in highest biocontrol level of 68% galling reduction. Single inoculation of Bm reduced nematode infection significantly by 70% whereas the reduction caused by the single application of T30 was 25%. Except for the combination of Fe14 and T30, the combination of other antagonists did not result in significant reduction compared to that of individual application of each antagonist.

In experiment 2, treatment with both T30+Bm reduced galling severity caused by *M. graminicola* significantly by up to 55% whereas combinations of Fe14+T30 or Fe14+Bm slightly reduced nematode infection compared to that of the control (Table 6.2).

Table 6.2: Effect of single or sequential applications of *Fusarium moniliforme* (Fe14), *Trichoderma* (T30) and *Bacillus megaterium* (Bm) on *Meloidogyne graminicola* infestation to rice 7 weeks after sowing under glasshouse conditions.

Treatments	Experiment 1		Experiment 2	
	M. graminicola /g root	Reduction (%)	*M. graminicola* /g root	Reduction (%)
T30	62 d	33	95 b	51
Fe14+Bm+T30	49 b	47	-	-
Fe14+Bm	31 b	55	159 a	19
Fe14	37 bc	60	171 a	12
T30+Bm	37 bc	60	88 b	55
Fe14+T30	20 cd	68	175 a	10
Bm	28 bc	70	153 ab	22
Control	93 a	-	195 a	-

Columns having the same letters are not significantly different based on the LSD test ($p \leq 0.05$, n=7).

Single application or sequential combination of the three antagonists did not significantly influence the growth of rice (Table 6.3). In most treatments, inoculations of biocontrol agents slightly increased rice root and shoot weights. Rice treated with both Bm+Fe14 had significant higher root weight and slightly higher shoot weight compared to that of control. The same tendency was observed in single treatment of Fe14 and combination of Fe14+T30. However, none of the treatments affected rice shoot height.

Table 6.3: Effect of single or sequential applications of *Bacillus megaterium* (Bm), *Fusarium moniliforme* (Fe14) and *Trichoderma* (T30) on growth of rice 7 weeks after planting under glasshouse conditions.

Treatments	Root weight (g)	Shoot weight (g)	Shoot height (cm)
Fe14	1.07 bc	1.04 ab	36.2
Bm	0.92 abc	1.24 ab	35.9
T30	0.57 a	0.94 a	35.6
Fe14+Bm	1.23 c	1.18 ab	32.8
Fe14+T30	1.03 bc	1.11 ab	34.2
T30+Bm	0.78 ab	1.21 ab	35.3
Fe14+Bm+T30	1.00 abc	1.35 b	35.7
Control	0.63 ab	0.97 a	33.7
P value	0.000	0.000	ns

Columns having the same letters are not significantly different based on the LSD test ($p \leq 0.05$, n=7).

4. Discussion

4.1 *In vitro* compatibility of Fe14, T30 and Bm

Compatibility of different antagonists amongst one another is an important factor when different biocontrol organisms are combined in a treatment system. In this experiment, growth rate of T30 when alone in 100% PDA was fastest. The growth of Bm did not exceed 3 mm of the original streak on culture medium which was expected for a bacterium. Slow growth of Fe14 in the presence of *Trichoderma* in the same plate suggested a slight inhibition effect of T30 on this isolate. *Trichoderm*a T30 probably produced antimicrobial compounds that influenced the growth of Fe14. However, T30 did not overgrow or cover mycelia of Fe14 but a deadlock zone was produced. This phenomenon was the most common interaction between two species cultured in the same plate. Deadlock also occurred between colonies of the same fungus as in the case of Fe14+Fe14 and this interaction is referred to as self-inhibition. Thus, a general judgement on compatibility of *Trichoderma* and the endophytic fungus Fe14 and the bacterium Bm from the dual culture test was difficult. The *in vitro* inhibition activity was tested *in vivo* in the following sections to validate the *in vitro* compatibility and investigate their biological interactions because Fe14 and Bm are endophytes while *Trichoderma* was previously demonstrated to be present only in the rhizosphere and thus, they occupy different niches. In addition, their different modes of action, if compatible would also improve nematode biocontrol efficacy.

4.2 Multiple applications of antagonists with different modes of action at sowing

The isolates Fe14 and T30 when applied alone or in combination significantly reduced *M. graminicola* infestation to rice. However, combination of Fe14 and T30 did not lead to greater biocontrol efficacy compared to single treatment of T30. Biocontrol levels ranging from negative over neutral to positive were reported in combinations of different biocontrol agents in controlling plant parasitic nematodes. Some researchers observed greater effects when combining *Furasium* and *Trichoderma* species in a biocontrol system. For examples, zum Felde et al. (2006) reported that inoculation of the endophytic *Fusarium* and *Trichoderma* isolates to banana suckers to control *R.*

similis improved biocontrol level significantly when compared to individual treatments under field conditions. Similarly, Mendoza and Sikora (2009) demonstrated greater effects when the endophyte *F. oxysporum* Fo162 was combined with *Paecilomyces lilacinus* in controlling *Radopholus similis* in banana. In their study, *F. oxysporum* showed induced systemic resistance while *P. lilacinus* was the nematode egg-pathogen. Diverse modes of action are likely to result in greater biocontrol efficacy when the antagonisms are applied in combination (Gutsy et al., 2001; de Boer et al., 2003).

In contrast, combination of some biocontrol agents might reduce overall biocontrol. For example, Meyer et al. (2001) demonstrated that combinations of *Burkholderia cepacia* and *Trichoderma virens* did not result in greater biocontrol level than the single treatment. The reason for this decreased effect was suggested to be due to the antagonism of biocontrol agents to each other. In comparison, the combination of *T. virens* and *B. cepacia* to control fungal diseases in corn, tomato and pepper was reported to improve control over that obtained when each agent was applied alone (Mao, 1998a, b). Masadeh et al. (2004) also obtained the same tendency when combining the AMF fungus *Glomus intraradices* and *T. viride* in biocontrol of root-knot nematodes in tomato. The combination of these two fungi however did not result in synergistic effects toward *M. hapla* and *M. incognita* in greenhouse experiments. The reason for this lower effect was believed to be due to the antagonism of biocontrol agents to each other.

In the present experiment, the combination of *F. moniliforme* and *Trichoderma* reduced galling severity significantly but it was not better than the single treatment, suggesting that combination would not improve the level of biocontrol.

Previous studies have demonstrated that individual applications of *Trichoderma*, *F. moniliforme* or *B. megaterium* reduced the penetration of *M. graminicola* into rice roots (Padgham and Sikora, 2007; Le et al., 2009). In this study on simultaneous application, all single and combined applications of these three antagonists reduced nematode infestation significantly by 20-38%. The only exception was the combined treatment Fe14+T30, where the effect was not significantly different from the control.

Many studies have been conducted on the effect of combining antagonists with different modes of action against plant pathogens (Meyers and Roberts, 2002). However, the degree of success varies substantially. While many combined treatments have resulted in greater effects (de Leij et al., 1992; Guetsky et al., 2001; Meyer et al.; zum Felde et al., 2005; Mendoza and Sikora, 2008; Chaves et al., 2009), some actually reduced the level of biocontrol when compared to single treatment (Esnard et al., 1998; de Boer et al., 1999; Meyer et al., 1998; Chen et al., 2000).

Scientists prefer a control system where antagonistic activity is optimized. Since the soil is a complex ecosystem where many organisms reside and interact with one another, combination of biological agents is believed to be a good option to protect the plants in different stage of development through different modes of action.

De Leij et al. (1992) demonstrated synergistic activity when the egg pathogenic fungus *Pochodia chlamydosporia* (syn. *Verticilium chlamydosporium*) and the bacterial parasitic *Pasteuria penetrans* were combined in soil in controlling *M. incognita* in tomato. A combination of the two agents caused the largest reduction in galling. Similarly, Mendoza and Sikora (2009) reported the same effect when the egg pathogen *Paecilomyces lilacinus* isolate 251 was combined with the endophyte *F. oxysporum* Fo162 and the pathogenic bacterium *B. firmus* to control *R. similis* in banana. The biocontrol level was improved when more antagonists were combined but not synergistically. However, in the present study, the combination of biocontrol agents did not result in greater level of biocontrol compared to individual treatments. This was probably due to competition for nutrients and space inside the root zone between Fe14 and Bm or the antimicrobial effect of T30 on the other antagonist as observed in the *in-vitro* dual culture test. Combinations may not be beneficial, as antagonism can occur between biocontrol organisms, and lead to unchanged control levels (Zaki and Maqbool 1991, Viaene and Abanoi 2000) or even to lower control (Esnard et al. 1998, Chen et al. 2000), when compared to individual applications of biocontrol agents.

4.3 Sequential application of Fe14, T30 and Bm

In this study, antagonists were combined in a staggered time manner, Fe14 and Bm at sowing and before transplanting and T30 incorporated with *M. graminicola* eggs. *Trichoderma* species are known as egg pathogens of wide range of plant parasitic nematodes and the biocontrol activity of *Trichoderma* has been extensively studies and can be considered one of the best studied biocontrol agents (Meyer et al., 2001; Masadeh et al., 2004; zum Felde et al., 2006; Dababat, 2007). In this set of experiments, *Trichoderma* spores and eggs of *M. graminicola* were mixed in the soil and left for a period of 2 weeks so that *Trichoderma* could infect the nematode eggs in the plant-free soil. Fe14 and Bm as endophytes need a certain period of time to colonize and establish inside the root zone for example after applied to rice seedbeds. By doing this, the effect of each antagonist against *M. graminicola* in and outside the root zone might be optimized. The experimental results showed that the combination of Fe14 and T30 significantly reduced nematode infestation by 68%. Other combinations of Fe14+Bm, Bm+T30 also significantly reduced galling severity compared to the control. However, the difference was not significant among these treatments. These results are similar to those obtained in the previous experiment in the section 3.2 in which no additive or synergistic effects were observed in combined applications of the antagonists. Mendoza and Sikora (2009) demonstrated that sequential combination of *P. lilacinus* PL 251 and endophytic *F. oxysporum* Fo162 significantly reduced nematode penetration compared to the single inoculation but the activity was not additive or synergistic. Likewise, zum Felde et al. (2006) illustrated that multiple combinations of endophytic *F. oxysporum* and *Trichoderma* resulted in significant reduction of *R. similis* penetration in banana. Other authors also applied multiple control agents and obtained improved control. In comparison, Meyers et al. (2001) did not observe greater biocontrol efficacy when they combined *P. fluorescens*.

It was suggested that antagonism between biocontrol agents may occur. Sequential application of *Trichoderma* T30 and *B. megaterium* Bm also reduced nematode infestation significantly in the present test. However, the combined inoculation was not

better compared to single treatment. Therefore, single treatments of each biocontrol agent were adequate.

5. Conclusion

The biological control activity of individual or combined applications of the endophytic *F. moniliforme* Fe14 with *Trichoderma* T30 and the endophytic bacterium *B. megaterium* Bm was investigated. From the experimental results the following conclusions can be made:

1) A single application of Fe14, T30 and Bm was sufficient to reduce *M. graminicola* infection significantly

2) Simultaneous applications of Fe14 with T30 and Bm did not lead to significant increase in level of biocontrol and thus is not recommended

3) Similarly, combined applications of Fe14 with T30 and Bm in different time manner are not recommended due to the non-significant results compared to individual treatments

SUMMARY AND RECOMMENDATIONS

Investigations on biological control of the root-knot nematode *M. graminicola* in rice are poorly documented. Therefore, the findings of the present research are particularly important and can be summarized as follow:

1) The endophytic *F. moniliforme* isolates Fe14 and Fe14 extensively colonized rice roots under both oxic and anoxic soil conditions and persisted in the root tissues over a 5 months period at a strong level.

2) Neither Fe1 nor Fe14 displayed pathogenic symptoms after 5 months exposure nor promoted rice growth.

3) Fe14 reduced penetration of the nematode to the rice roots but also reduced nematode development and reproduction.

4) Root exudates from Fe14 treated plants altered host finding ability of *M. graminicola* indicating physiological changes in the rice root or the release of toxic substances.

5) Induced systemic resistance was demonstrated for the first time in rice with an endophyte in a split-root system design. Biocontrol therefore resulted from initiation of induced systemic resistance coupled with changes in host finding ability.

6) Fe14 exhibited high levels of biocontrol under anoxic soil conditions. The findings demonstrated for the first time the potential of applying endophyte biological control technology in different soil water regimes.

7) Seed treatment as well as soil drenching was demonstrated to be effective methods of fungal inoculation for successful endophytic colonization and biocontrol.

8) Single application was adequate to achieve high level of biocontrol.

9) The endophyte Fe14 isolate requires an incubation period to colonize endorhiza and improve establishment before biocontrol can take place. Therefore seed treatment combined with seedbed production is recommended for optimum use of this biocontrol system.

10) Combinations of the endophyte Fe14 with different biocontrol agents such as the egg pathogen *Trichoderma* or the endophytic bacterium *Bacillus megaterium* did not lead to greater levels of biocontrol compared to individual treatments and thus are not recommended.

11) The present study not only demonstrated great potential of applying biocontrol technology against *M. graminicola* in rice but also leads to the possibility of developing a model of biocontrol system against other important pests under different soil water regimes.

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