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Association of arbuscular mycorrhizal fungi and *Crotalaria spectabilis to* control the burrowing nematodes (*Radopholus similis*) in banana

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Docteur en Sciences agronomiques et ingénierie biologique

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List of abbreviations

AMF: Arbuscular mycorrhizal fungi

ANOVA: Analysis of variance

BCA: Biological control agent

CIRAD: Centre de coopération internationale en recherche agronomique pour le développement

ERM: Extraradical mycelium

FAO: Food and Agriculture Organization of the United Nations

FAOSTAT: Statistics Division of the FAO

GINCO: Glomeromycota *in vitro* collection

H-AMP: half-closed arbuscular mycorrhizal plant

HC: Hyphal compartment

ICIPE: International Centre of Insect Physiology and Ecology

IITA: International Institute of Tropical Agriculture, Nigeria

INIBAP: International Network for the Improvement of Banana and Plantain

IPBO: International Plant Biotechnology Outreach

IPGRI: International Plant Genetic Resources Institute

ISR: Induced Systemic Resistance/tolerance

JA: Jasmonic Acid

MDP: Mycelium donor plant (*in vitro* culture system)

MIR: Mycorrhiza Induced Resistance/tolerance

MS medium: Murashige Skoog medium

MSR medium: Modified Strullu-Romand medium

MUCL: Mycotheque de l’Universite catholique de Louvain

NRCB: National Research Centre for Banana, India

PGPR: Plant Growth Promoting Rhizobacteria

PIF : Plant Issu de Fragment de tige de bananier

PPF : Photosynthetic Photon Flux

PROMUSA : Programme Mondial pour l'amélioration des Musa

RC: Root Compartment

RH: Relative Humidity

ROC: Root organ culture

Glossary

Arbuscular mycorrhizas: “Widespread type of endomycorrhizal interactions involving fungi of the phylum Glomeromycota, the hyphae of which reach the root inner cortex and develop highly branched exchange structures called arbuscules” (Bonfante and Genre, 2010).

Arbuscule: “Highly branched structure produced by arbuscular mycorrhizal fungi inside the cell lumen of their host. Arbuscules are considered to be the key elements of the symbiotic nutrient exchanges between the plant and the fungus” (Bonfante and Genre, 2010).

Chemotaxis: Movement of cells or organisms in response to chemicals, whereby the cells are attracted (positive chemotaxis) or repelled (negative chemotaxis) by substances exhibiting chemical properties (MediLexicon, 2018).

Extraradical mycelium: “Hyphal network that develops in the rhizosphere, in which it absorbs inorganic nutrients that are transferred to the host plant through intraradical hyphae” (Bonfante and Genre, 2010).

GRIN: Germplasm Resources Information Network

Intraradical hyphae: “Network of hyphae from mycorrhizal fungi that colonizes the host root tissues” (Bonfante and Genre, 2010).

Inoculation: “application of microorganisms’ inoculum to plant seeds or into soil ready for planting”.

Inoculum: a portion of an organism which is transferred to a host and is capable of infecting it. It may consist of spores, bacteria, mycelial fragments or virus particles.

Induced systemic resistance/tolerance: “Enhanced defensive capacity of the entire plant against a broad spectrum of pathogens; acquired upon local induction by beneficial microbes” (Pieterse *et al.*, 2014).

Micropropagation “the practice of rapidly multiplying stock plant material to produce a large number of progeny plants under aseptic conditions using modern plant tissue culture methods” (Singh *et al.*, 2011).

Microcosm: compartmented culture pot divided above-ground an below-ground in compartments to avoid roots of differents plants in the system to intermingle.

Mycorrhiza induced resistance/tolerance: ‘‘a root colonization by AM fungi improving plant resistance/tolerance/tolerance to biotic stresses’’ (Pozo *et al.*, 2009)

Mycorrhizal plant: plant (species) able to form mycorrhizal symbiosis.

Mycorrhizas: “plant-fungal symbioses that are typically mutualistic, obligate and based on an exchange of photosynthates for soil minerals” (Boostani *et al.*, 2014).

Mycorrhized plant: plant living in association with a mycorrhizal fungus.

Poor host: “A poor host to a nematode species is a plant in which the nematode has low increasing of its population”. **- Non host**: “A non-host to a nematode species is a plant in which the nematode fails to reproduce” (Wang *et al.*, 2002).

Push pull: “The push pull strategy is a strategy for controlling agricultural pests by using repellent "push" plants and trap "pull" plants” (Cook *et al*., 2007).

Resistance/tolerance: “The criteria for host plant resistance/tolerance are 1) failure of the nematode to live inside the host or early nematode death in the host, 2) decreased production of eggs, or 3) inhibition of nematode growth or development” (Wang *et al.*, 2002).

Viability: In this context nematodes viability was observed when they are completely immobile after their bathing in root exudates solutions.

Summary

Banana belongs to the most consumed fruit worldwide. Unfortunately, its production is threatened by numerous pests such as nematodes. Under conventional production systems, nematodes are most often controlled by nematicides. These chemicals may have side effects on human health and the environment, requiring the development of alternatives control measures. Among these are the use of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) and push pull plants such as *Crotalaria spp*. Here we investigated the combination of AMF with *C. spectabilis* as control measure against nematodes in banana.

Three objectives were pursued in this thesis. First, we studied the effects of AMF on nematodes population and root infestation of banana plantlets grown under autotrophic *in vitro* culture conditions. Secondly, we investigated the impact of AMF and *C. spectabilis* together on the control of *Radopholus similis* population in banana. Finally, we explored the impact of roots exudates of AMF-colonized *C. spectabilis* plantlets on the movement and vitality of *R. similis*.

Whatever the experiment, all the developmental stages of the nematodes were affected in presence of the AMF. The multiplication rates of the nematodes were drastically decreased, while no effects of the nematodes were noticed on the AMF. Also *C. spectabilis* decreased the nematode population in banana roots, although more slightly. Interestingly, the effects on nematodes were even more pronounced when the AMF and *C. spectabilis* were considered together. Indeed, the concomitant use of AMF and *C. spectabilis* impacted all the developmental stages of the nematodes and significantly decreased the surface of necrotic roots as compared to both organisms alone. Concerning the mobility, nematodes were more attracted by exudates of *C. spectabilis* when opposed to banana exudates suggesting the presence of attractant compounds. The vitality of nematodes was affected by the root exudates of *C. spectabilis*, while they remained mobile in exudates of banana roots. This effect was highly concentration-dependent and nematodes recovered mobility after cleaning from the *C.*

spectabilis exudates suggesting a nematostatic rather than nematicidal effect of the exudates. The impact of AMF on exudates composition could not be ascertained nor excluded.

In conclusion, we confirmed the effects of AMF on the control of nematodes populations and root damage in banana. Their combination with a push pull plant (*C. spectabilis*) increased even more this effect by reducing drastically the surface of necrotic roots in bananas. Therefore it suggests that the combination of both organisms may be an innovative strategy in integrated pest management, although field trials need to be conducted to confirm this statement. Finally, AMF may possibly modify the exudates of *C. spectabilis* and thus exert an indirect effect on nematodes, although this needs as well to be demonstrated.

Outline of the thesis

The PhD thesis is structured as follows (**Figure 1)**: introduction – state of the art – research objectives – materials and methods – research results – general discussion – conclusion and perspectives.

In the **introduction** section, the context and the general objective of the study were presented. In the **State of the art** section, the most recent and pertinent literature on banana, nematodes, AMF and crotalaria were reviewed. The importance of banana as a staple crop and the major constraints impacting its production, with a particular attention to the burrowing nematode *Radopholus similis*, were approached. The management of this pest was reviewed with a special attention to AMF and push pull plants like *Crotalaria spectabilis*. The **Materials and Methods** section detailed the biological materials considered and briefly described the most important culture systems used throughout the study and analysis methods applied.
The objectives of the thesis are further presented in the **Research objectives** section.
In chapter 1 we investigated the effects of AMF on nematodes population and root infestation on banana plantlets grown under autotrophic *in vitro* culture conditions.
The results were published in Journal of Nematology (Anene *et al.*, 2013).
In chapter 2 we investigated the impact of AMF and *C. spectabilis* together on the control of *R. similis* in banana.
The results were published in the journal of Applied Soil Ecology (Anene *et al.*, 2016).
In chapter 3, we investigated the impact of roots exudates of AMF-colonized *C. spectabilis* on the movement and viability of *R. similis*.

The results are submitted to Mycorrhiza for publication.

In the **General Discussion** section, the major findings of the thesis were summarized and discussed. Finally, in the **Conclusions and Perspectives** section, the major outcomes of the thesis were given and commented. Further studies to deepen our understanding on the nematodes control mechanisms via AMF and *C. spectabilis* were suggested.

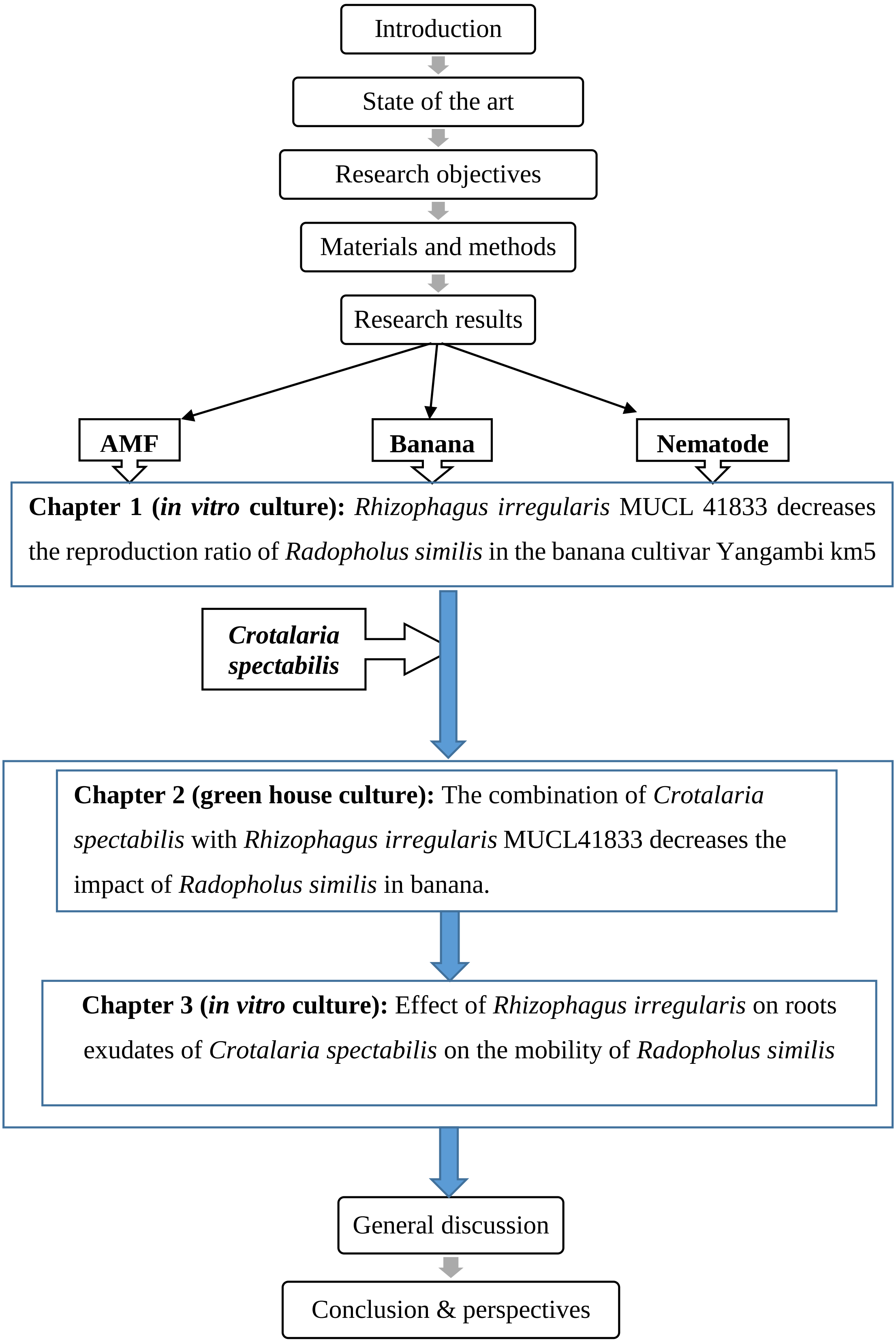

Figure 1: Research outline of the thesis

Authors contribution

The **Introduction** and **State of the art** (not published) as well as the **General Discussion**, **Conclusions** and **Perspectives** were written by myself.

Chapter 1 was fully designed and conducted by myself; coauthors of the chapter have been involved in giving the introduction to the in vitro culture of AMF and in revising the manuscript. My contribution to this chapter was estimated to 85%.

In **Chapter 2**, the design of the experimental procedures, the practical work and data analysis were performed by myself. The statistical analysis was performed with the assistance of Alain Guillet, SMCS - IMMAQ at UCL.Coauthors of the chapter have been involved in revising the manuscript. My contribution to this chapter was estimated to 90%.

In **Chapter 3,** the design of the experimental procedures, the practical work and data analysis were performed by myself. The statistical analysis was performed with the assistance of Alain Guillet, SMCS - IMMAQ at UCL. Co-authors of the chapter have been involved in revising the manuscript. My contribution to this chapter was 85%.

I. INTRODUCTION

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Banana is a major staple food for rural people in sub-Saharan Africa, Latin America and Asia (Lassoudière, 2011; Shobhana *et al.*, 2014; CIRAD, 2014). It represents a major income source for many countries, is commercialized in rural markets and exported worldwide (Shobhana *et al.*, 2014; CIRAD, 2014). Unfortunately, its production is threatened by shoot, bunch and root pests and diseases (INIBAP, 2001; Ploetz, 2003; AUGURA, 2009). Among the root pests, nematodes (e.g. *Radopholus similis*, *Pratylenchus coffeae*, *Meloïdogyne incognita*) are particularly problematic (Quénehervé, 2009; Hölscher *et al.*, 2014). For instance, the migratory nematode *R. similis* (the most common in banana plantations) feeds on the root cells and forms large cavities while migrating in the cortical parenchyma (De Buck and Swennen, 2016) which then coalesce into necrotic tunnels (Quénehervé, 2009). The consequences are a weakened root anchorage with toppling of plants, decreasing yield by 30 to 75% in the worse cases (Quénehervé, 2009).

Usually, nematodes are controlled with nematicides. However, because of their adverse effects on the environment and human health, many of these chemicals have been banned. This has encouraged the development of other control measures such as fallow, paring and hot water treatment of the corms, the use of resistant cultivars and the planting of *in vitro* produced plantlets (Quénehervé, 2009).

The application of bio-control organisms (e.g. nematophagous fungi and bacteria such as *Arthrobotrys irregularis*, *Paecilomyces lilacinum*, *Pasteuria penetrans*) is an alternative option increasingly considered since the last two decades to decrease the pressure caused by nematodes (e.g. Siddiqui and Mahmood, 1996, 1999; Duponnois *et al.*, 1998). Another group of organisms is receiving an increasing attention for the biocontrol of nematodes, i.e. the arbuscular mycorrhizal fungi (AMF) (Azcón-Aguilar and Barea, 1996).

Arbuscular mycorrhizal fungi are key soil microorganisms that form symbiotic associations with the vast majority of land plants, improving their growth and resistance/tolerance against abiotic and biotic stresses (Smith and Read, 2008). Several studies have been conducted in pots as well as under *in vitro* culture conditions to evaluate their potential in decreasing the incidence caused by nematodes (Azcón-Aguilar and Barea, 1996, Vos *et al.*, 2012; Koffi *et al.*, 2013; Lopez and Sword, 2015; Panebianco *et al.* 2015). In particular, *in vitro* culture of root organs (named root organ culture – ROC) colonized by AMF have been used as a contaminant-free model system to study the tripartite interaction AMF / root / nematode. For instance, Elsen *et al.* (2001a, 2003) observed a decrease by half of the populations of *R. similis* and *P. coffeae* in AMF-colonized ROCs of carrot. However, these studies were conducted on excised transgenic roots (i.e. in the absence of photosynthetic tissues), and therefore in absence of a true source-sink (Fortin *et al.*, 2002). More recently, Koffi *et al.* (2009) developed an *in vitro* cultivation system associating autotrophic *in vitro* produced banana plantlets with AMF. This system offers large perspectives for studying the tripartite system AMF / plant / nematode and therefore to investigate the role of AMF in the resistance/tolerance conferred to banana against phytopathogenic nematodes.

Several studies have also reported the role of push pull plants (e.g. marigold, *Crotalaria species*) in the bio-control of pests. *Crotalaria* is a leguminous plant cultivated as cover crop for weed control (Wang and McSorley, 2012). Many species of *Crotalaria* are grown as green manure to increase nitrogen content in the soil via their symbiosis with rhizobium (Wang *et al.*, 2002). Remarkably, *Crotalaria* species also produces allelochemicals that are toxic or inhibitory to some pests and provide a niche for antagonistic flora- and fauna-trapping nematodes (Wang *et al.*, 2002). Indeed, a number of studies (Wang *et al.*, 2002; Thoden *et al.*, 2009) have reported that *Crotalaria* species are non-host for root-rot nematodes and poor hosts for some migratory nematodes such as *R. similis*. Interestingly, Germani and Plenchette (2004) reported that *Crotalaria sp*. are highly mycotrophic plants and suggested that they could be used as pre-crops for providing green manure while at the same time decreasing the number of detrimental nematodes and increasing the level of beneficial AMF. *Crotalaria sp.* could thus potentially be used as AMF-mycelium donor plants (MDP) for the cash-crop (e.g. banana) because of its capacity for fast mycorrhization and its aptitude to provide a dense mycelium network within few weeks.

Within this thesis, we aimed to investigate the combination of the AMF *Rhizophagus irregularis* MUCL 41833 and the push pull plant *C. spectabilis* for the control of *R. similis* in banana. In a first step, conducted *in vitro*, we completed a study investigating the effects of the AMF *R. irregularis* on *R. similis* in banana (c.v. Yangambi km5 and Grande naine). The effects of the AMF on the reproduction ratio of the nematodes as well as the impact of the latter on the AMF were monitored. Secondly, we investigated the combination of *R. irregularis* with *C. spectabilis* on the population of *R. similis* in banana plantlets (c.v. Grande naine) grown under greenhouse conditions. Root colonization by the AMF and infection by the nematodes were monitored in presence/absence of *C. spectabilis* to evaluate the impact of the AMF, *C. spectabilis* and both in combination on the nematodes and the impact of the later on the fungal symbiont and push pull plant. Finally, we studied the effects of roots exudates of AMF-colonized or non-colonized *C. spectabilis* plantlets as attractant or repellent of *R. similis* by investigating nematode chemotaxis in a gelled medium supplemented with the exudates.

II. STATE OF THE ART

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2.1. Banana

2.1.1. Origin and Taxonomy

Bananas are native to South East Asia where they have been cultivated for thousands of years. They were introduced to Africa in prehistoric times (Crane *et al.*, 2008) and further transported to the New World (i.e. Ecuador) around 200 B.C. by the first explorers and finally by missionaries to the Caribbean (Karthikeyan and Kalaiyarasu, 2014). From there, bananas were spread under the tropics and the warm subtropics. In the 3rd century B.C., edible bananas were consumed in the Mediterranean region but awaited the 10th century A.D. to be consumed in Europe (Morton, 1987).

Bananas belong to the Musacaea family and genus *Musa*. There are two species of banana, *Musa acuminata* and *M. balbisiana* (Crane *et al.*, 2008), which fruits bear non-digestible seeds so that they are non-consumable. Almost all modern edible bananas originate from these two seed-producing ancestors. Spontaneous cross-pollination between wild varieties of *M. acuminata* and/or *M. balbisiana* led to seedless edible bananas plants (De Buck and Swennen, 2016).

Edible bananas can be divided in two main categories; the dessert bananas and the non-dessert bananas including cooking (plantain) and beer bananas (Quénehervé, 2009; De Buck and Swennen, 2016). Dessert bananas have sweet fruits that are eaten raw when ripe. Plantains are generally longer and have a thicker peel than dessert bananas and are eaten as a vegetable (De Buck and Swennen, 2016). They are cooked ripe or not and sometimes, the ripe fruits are eaten raw.

Genetically, bananas are grouped depending on the number of chromosomes and their origin (De Buck and Swennen, 2016). Bananas from the ancestor *M. acuminate* have A-genome and from *M. balbisiana* B-genome (Fig.2). Most edible bananas are triploids with AAA-genomes (dessert bananas), AAB-genomes (plantain and some dessert bananas) and ABB-genomes (cooking bananas) (De Buck and Swennen, 2016).

Banana is a monocotyledonous herbaceous fast-growing plant consisting of one or more pseudostem (upright, trunk-like structure) formed by tightly packed concentric layers of leaf sheaths (Fig. 3), an underground rhizome, and a fibrous root system (Crane *et al.*, 2008). The pseudostem constitutes the functional trunk which supports the leaves and the flower and fruits bearing stalk. Its optimum growth temperature is 26 - 28°C and it prefers flat and well drained soils that are high in organic matter with a pH of 5.5 – 7.0.

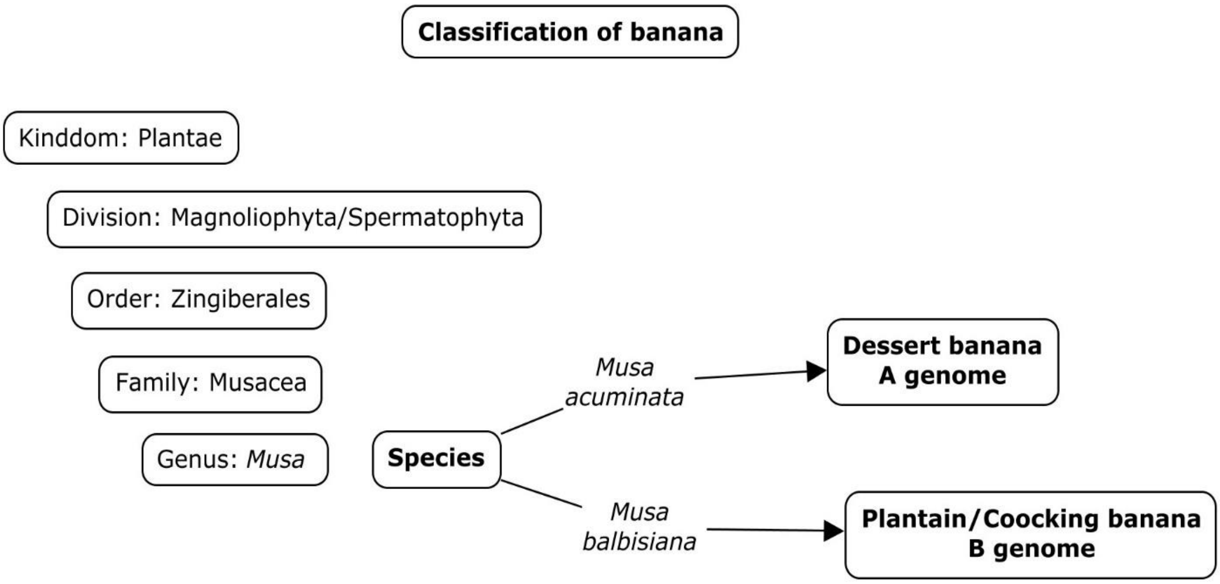


Figure 2: Classification of banana (GRIN, 2009)

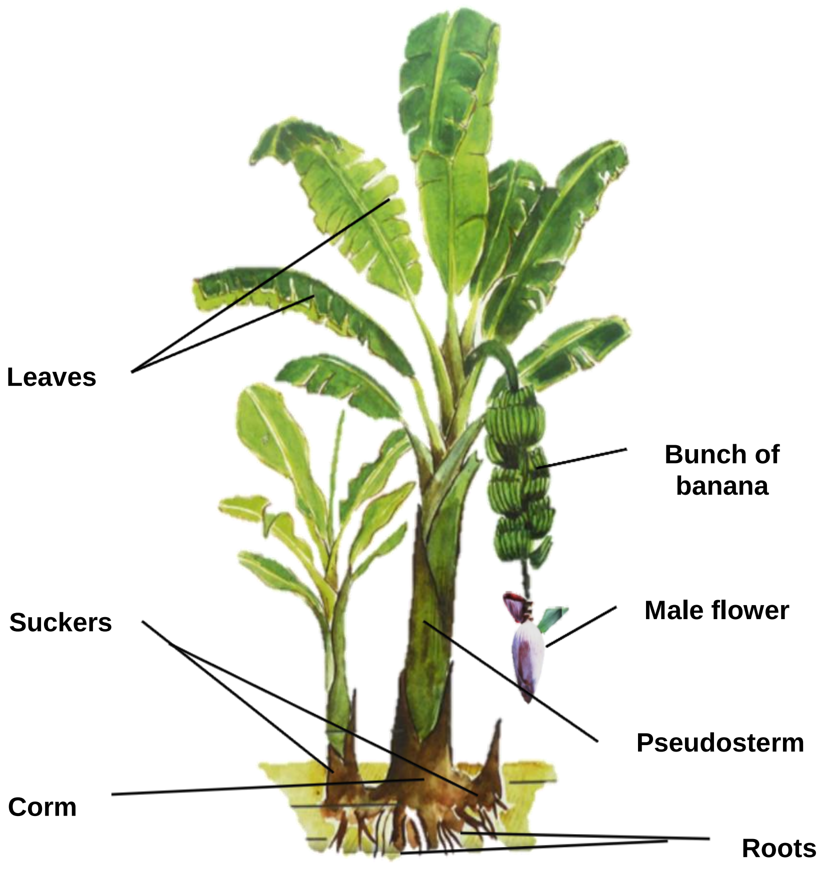


Figure 3: Banana plant (Anene, 2016)

2.1.2. Economic importance

Large-scale production of banana started in 1834 and increased sharply in the late 1880's (Karthikeyan and Kalaiyarasu, 2014). It is a major staple food (rich in carbohydrate) for rural people in sub-Saharan Africa, Latin America and Asia and represents one of the major income sources for many countries (Lassoudière, 2011; Shobhana *et al.*, 2014; CIRAD, 2014). In 2016 around 112 millions of tons of bananas were produced with 18 millions of tons for exportation (trade and shipping) (FAO, 2018; CIRAD, 2018). The production is focused on two main types of bananas; (1) dessert bananas, among which the Cavendish subgroup, mainly produced for export, and (2) cooking bananas, principally plantains (Risède *et al.*, 2010a). The export of banana represents 7 billions of dollars yearly. Asia countries (India, China, Philippines and Indonesia) are the leading producers of banana in the world followed by Latin America (Brazil, Ecuador, Guatemala) and Africa (Angola, Tanzania and Burundi) (FAO, 2018; CIRAD, 2015). The top 3 first countries suppliers are Ecuador, Philippines and Costa Rica (CIRAD, 2015).

2.1.3. Production constraints

Bananas production is threatened by numerous pests and diseases. Since they are produced by vegetative multiplication and are thus clonal, once a variety is exposed to a disease or pest, it spread all over the plants with yield losses occurring very rapidly (De Buck and Swennen, 2016). Above as well as below-ground parts may be affected. Above-ground, the plant is often threatened by fungal (e.g. Panama diseases, Fusarium wilt, black and yellow sigatoka caused by *Mycosphaerella spp*.), viral (e.g. Banana Bunching Top Virus - BBTV and Banana mosaic Streak Virus – BSV) and bacterial (e.g. disease of moko caused by *Ralstonia solanacearum,* Banana Bacterial Wilt Disease caused by *Xanthomonas campestris* pv. *musacearum*) diseases (CIRAD, 2014). Below-ground, the root systems and corm may be affected by pests and diseases such as the banana weevil (*Cosmopolites sordidus*), various nematodes (e.g. *R. similis, P. goodeyi*, *P. coffeae, Helicotylenchus multicinctus* and *Meloidogyne spp*.), fungi (e.g. *Fusarium oxysporum, Cylindrocladium spathiphylli*) and bacteria (e.g. *Erwinia* species). Overall, it is admitted that fungal diseases may decrease yield by 30 to 70%; viral diseases by 50 to 100% and nematodes by 30 to 70% (Quénehervé, 2009; De Buck and Swennen, 2016).

2.2. Banana parasitic nematode: *Radopholus similis* (Cobb, 1893) Thorne, 1949

Plant-parasitic nematodes are tiny microscopic worms that live mainly in soil and roots. In the case of banana plants, the most damaging species spend most of their life cycle in root and corm tissues (Risède *et al.*, 2010a). Nematodes usually found in banana plantations are migratory nematodes (i.e. *R. similis, P. goodeyi, P. coffeae, H. multicinctus* and root-knot nematodes (i.e. *Meloidogyne spp*.) (De Buck and Swennen, 2016). Among them, particular attention will be addressed to the burrowing nematode *R. similis* (Fig. 4 and 5), one of the most common and damaging nematode in banana plantations.

R. similis is native to Australasia, but is found worldwide in tropical and subtropical regions of Africa, Asia, Australia, North and South America, and the Caribbean region (Sikora and Crow, 2012). The widespread presence of this nematode is mostly caused by its dissemination with propagation plant material, especially infested banana corms (O'Bannon 1977, Gowen *et al.*, 2005).

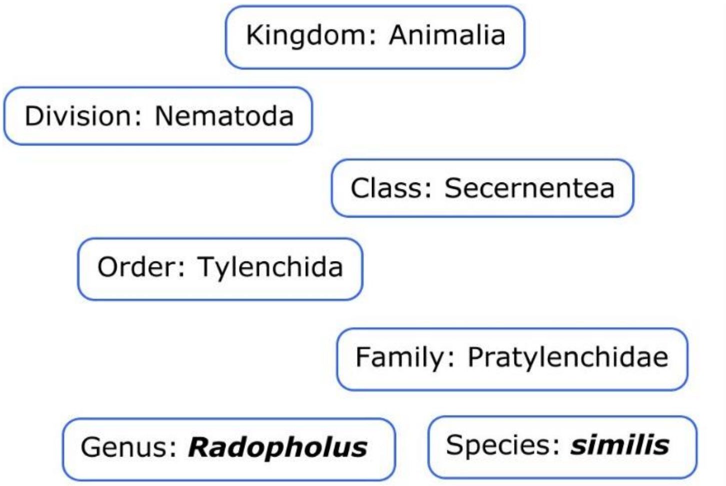


Figure 4: Classification of *R. similis* (Cobb, 1893; Thorne, 1949)

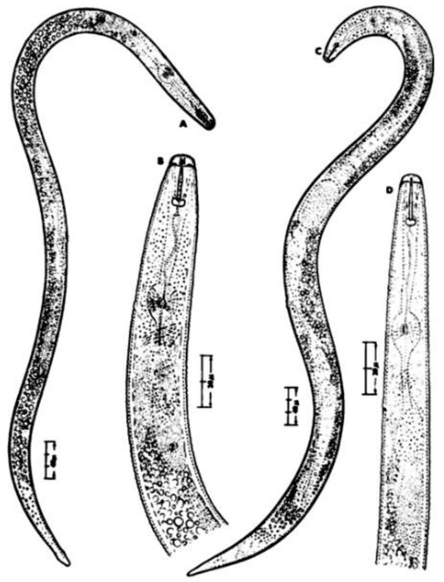


Fig. 1. Radopholus similis females. A) Young female. B) Anterior body of mature female. C) Mature female. D) Anterior body of young female.

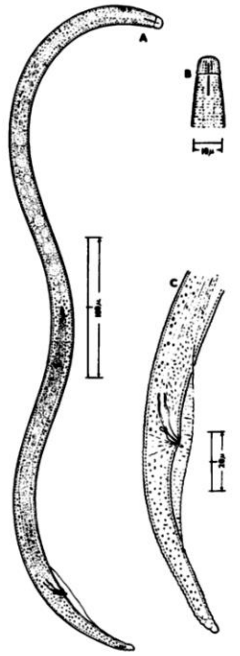


Fig. 2. Radopholus similis male. A) Entire body. B) Head. C) Tail.

Figure 5: Schema of *R. similis* (from Esser *et al.*, 1984)

2.2.1. Biology and life cycle of *R. similis*

Radopholus similis has a wide host range, infecting numerous crops belonging to families such as Musaceae (bananas), Poaceae (rice, maize), Liliaceae (onion), Fabaceae (pigeon peas, cowpea, ground nut), Cucurbitaceae (bitter gourd), Convolvulaceae (sweet potato), Solanaceae (tomato, chili) (Koshyk and Sosamma, 1975) and several ornamental plants (Sikora and Crow, 2012). Over 300 plants have been recorded as hosts of *R. similis* (Esser *et al.*, 1984; Sikora and Crow, 2012).

The spread of *R. similis* is done by rooted planting material, by soil containing nematodes, and, within a plot, by root contact or near contact, by irrigation water, agricultural machinery…

(Sikora and Crow, 2012). *Radopholus similis* can survive up to 5 years in soil without bananas, presumably because of alternative weed hosts.

Radopholus similis is an endoparasitic migratory nematode. Its development stages occurs within the host tissues, although in adverse conditions the nematodes may exit the roots.

All motile juvenile stages and females can infect root tissues at any point along the root. After root penetration, they mainly feed and migrate into the cortical parenchyma and also into the stele (Sikora and Crow, 2012). Mature males of burrowing nematode are not infective. As the mature females migrate through root tissue, they lay eggs that are produced through either sexual reproduction with males or by hermaphroditism (Thorne, 1961; Kaplan and Opperman, 2000). Once an egg hatches, the emergent second-stage juvenile can migrate within the root and complete its entire life cycle within the root system (Fig. 6), or it can leave the roots in search of another healthy host root (Sikora and Crow, 2012).

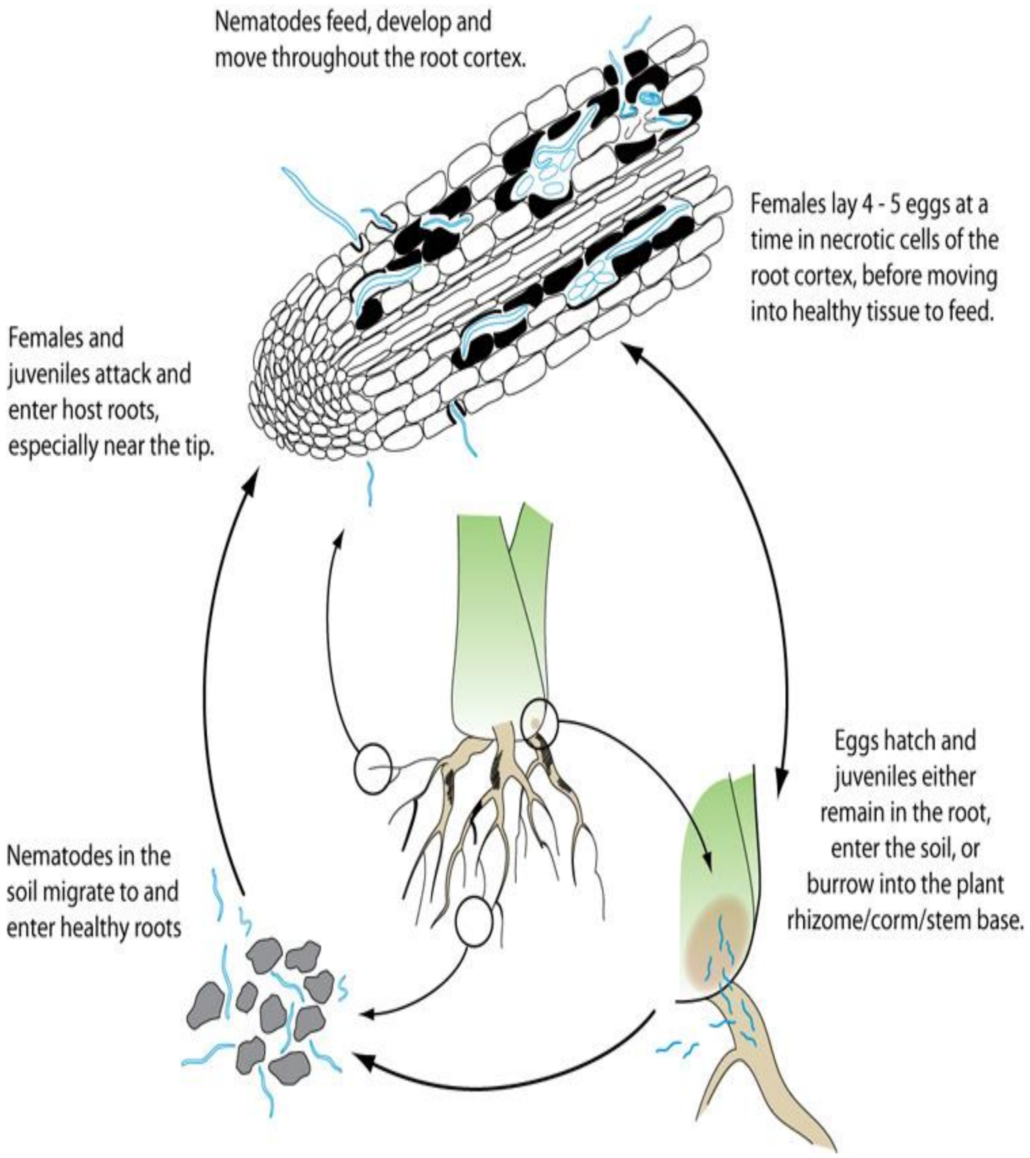


Figure 6: Life cycle of *R. similis* (Brooks, 2008)

The life cycle of *R. similis* in banana roots, from egg to egg, occurs in 20 to 25 days at 24 to 32°C. Eggs generally hatches in 8 to 10 days with juvenile stages completed between 10 and 13 days. There are four juvenile stages: the first stage (J1) develops within the egg, then molts and emerges from the egg as a J2 nematode, that further molts to third and fourth stages juveniles (J3 and J4) before the final molt to the adult stage (Hunt *et al.*, 2013).

2.2.2. Damages caused by *R. similis*

Radopholus similis is one of the major pest limiting banana production in several countries (Nair, 1979). It was reported to reduce banana yield by up to 41% in India, 33-76% in Africa (Quénehervé, 2009), 30-80% in Florida (Gowen *et al.*, 2005). It can infect root tissue at any point along the root and weaken the root system anchorage in the soil. It feed on cells and form large cavities while migrating in the cortical parenchyma (De Buck and Swennen, 2016) which then coalesce into necrotic tunnels (Quénehervé, 2009). Infected roots develop dark red streaks and necrotic lesions (Sikora and Crow, 2012). Then the lesions join together and turn black as they are invaded by other microorganisms (Speijer and De Waele, 1997). Finally rot appears and roots die. The corm of banana is also infected and show black areas at the infected points. Roots are destroyed. They can no longer feed the plant with water and minerals and provide them with strong anchorage in the soil. Consequently, the mother plant and suckers grow slow. The number and size of leaves are smaller than normal and becomes yellow. The fruit bunches are small. Finally, the plant fall down under heavy rain or winds before fruits are mature, a symptom known as "toppling" (Speijer and De Waele, 1997; Risède *et al.*, 2010a; Sikora and Crow, 2012).

According to Quénehervé (2009), it is possible to define three levels of nematode damage:

1. A lengthening of the banana vegetative phase: the different phenological intervals (period between planting and flowering, harvest and flowering of ratoons, harvest to harvest …) are lengthened without significant reduction in plant size, bunch weight, number of harvested bunches and total harvest. This minor damage is mostly ignored.
2. A lengthening of the banana vegetative phase with a reduction in yield: in this case there is a reduction in the number of harvestable bunches (bunches that are non-exportable because of

poor quality or immature delayed fruits) and in the average plant size and bunch weight. This type of damage is often observed in commercial plantations in West Africa.

3. A lengthening of the banana vegetative phase, with a reduction both in yield and in the longevity of the plantation: this third level is the same as above but is irreversible, due to the destruction of the plant which is uprooted or which growth is too severely delayed. When infested with the highly pathogenic strain of the burrowing nematode and in absence of any nematode control, this third level of damage is observed almost worldwide on dessert bananas.

2.2.3. Control strategies

2.2.3.1. Chemical control

In commercial banana plantations, nematode control mostly relies on chemicals (Risède *et al.*, 2010a). Between 1960 and 1978, fumigant nematicides were used extensively in Africa, Latin America and the Caribbean. Treatments are applied twice a year using hand-held injectors in which the fumigant is injected around individual plants (Quénehervé, 2009). Thereafter, research efforts have been oriented towards the formulation of non-fumigant nematicides such as organophosphates (Mocap, Nemacur) and carbamates (Vydate, Temik) mostly used as systemic or soil treatment after planting (Guerout, 1975; Quénehervé, 2009). These products were applied as granular or liquid formulations by hand-held applicators (workers). Almost all of these products have been gradually banned because of their toxicity to human beings and to the environment. They are nowadays, with a few exceptions, not used anymore. This resulted in the development of alternative strategies to manage nematodes populations: prophylactic, cultural practices and biocontrol agents.

2.2.3.2. Prophylactic strategies

Prophylactic strategies are based on the use of clean and resistant suckers or on *in vitro* produced plant material. *In vitro* produced banana plants represent an opportunity to use clean planting material. Such plants are recommended when new banana plantations are established. However, the cost of this planting material is not accessible to all farmers. That is why in Cameroon, the CARBAP developed an *in vivo*-plant production system based on the technology of PIF (Plantes issues de Fragment de tiges de bananiers (Kwa, 2003). The PIF banana plantlets are obtained by destroying the apical meristem to induce the activation of axillary buds.
Nematode-free banana corms could also be used. Hot-water treatment (immersion in water at 52-55 °C for 15-20 minutes, or boiling water for 30 seconds) following elimination of the lesions by peeling the corm (Quénehervé, 2009) is a standard practice in many traditional banana plantations under soils free of burrowing nematodes (Sikora and Crow, 2012).

Radopholus similis does not develop a resistant stage and its persistence in soils is thus limited. Soil sanitation, which aims at reducing the nematode population density in the soil, may thus represent an efficient practice to decrease population (Risède *et al.*, 2010a). In the French West Indies, improved fallow (based on the destruction of nematode-infested banana plots by injecting a reduced quantity of herbicide into the pseudostems (Risède *et al.*, 2010a)) and water isolation ditches, to delay the recontamination of fallows, are recommended. In this context, monitoring soil sanitation by potted biotests before banana planting is essential in commercial banana plantations. The principle is to trap and multiply nematodes present in soil samples using *in vitro* produced plants of a highly nematode-susceptible banana variety. Then the percentage of nematode-infested plants is estimated after two months of culture (Risède *et al.*, 2010a). To delay nematodes infestation in banana plantation, nematodes tolerant/resistant varieties associated to monitoring soil sanitation is an option. The selection of banana hybrids that are resistant to nematodes is a promising medium-term solution that has already been initiated by the CIRAD breeding program, and is currently developed in the framework of the 'Plan Banane Durable' (Risède *et al.*, 2010a). Dochez *et al.* (2006) reported in their study ten new potential sources of resistance/tolerance to *R. similis* within *Musa* diploids (AA) and triploids (AAA, ABB) from Papua New Guinea, Malaysia and the Philippines.

2.2.3.3. Cultural practices

Some cultural practices have been developed to manage nematode populations in banana plantations. Rotations with non-susceptible crops such as sugarcane (Loos, 1960; Risède *et al.*, 2010) or pineapple (Sarah, 1989) have been developed in the French West Indies. Weed fallow (Chabrier and Quénehervé, 2003) and cultivated fallow with Pangola grass (*Digitaria decumbens* - Stoyanov, 1967; Risède *et al.*, 2010b) and Sudan grass (Ternisien and Melin, 1989) have also been suggested. Other crops such as the pasture grass creeping signal grass (*Brachiaria humidicola*) and Guinea grass (*Panicum maximum*) and as legume grasses Stylo grass (*Stylosanthes hamata*) and Siratro (*Macroptilium atropurpureum*) (Risède *et al.*, 2010b) have been suggested.

In some cases, cover crops which are non-hosts for nematodes are used. Their association with bananas has been reported to increase the below-ground microbial biodiversity in banana cropping system, promoting the beneficial soil biota (Risède *et al.*, 2010b). Two crops are well developed in this cropping system in the French West Indies: *Impatiens spp.* (shade-tolerant Balsamisaceae which does not compete with banana) and *Neonotonia wightii* or perennial soybean (a legume with a strong tap root that penetrates vertically into the deep soil layers). Other cover crops such as *Crotalaria* species are receiving increasing attention nowadays (Wang *et al.*, 2002) (see section 2.4).

2.2.3.4. Biocontrol agents against nematodes

Biological control can be defined as the directed, accurate management of common components of ecosystems to protect plants against pathogens (Azcón-Aguilar and Barea, 1996). Biological control of plant pathogens is currently accepted as a key practice in sustainable agriculture because it is based on the management of a natural resource, i.e. certain rhizosphere organisms known to develop antagonistic activities against harmful organisms (bacteria, fungi, nematodes…) (Azcón-Aguilar and Barea, 1996). Biocontrol agents include parasitic fungi, rhizobacteria, mycorrhizal fungi and endophytic fungi (Sikora and Pocasangre, 2005; Nega, 2014) that are able to colonize permanently either the rhizosphere or the roots and to induce/promote directly or indirectly the natural plant defense against plant biotic constraints (Barea *et al.*, 2002).

Plant-parasitic nematodes have many natural enemies in the soil and early research on biological control focused mainly on microorganisms which are predacious (e.g. trapping fungi) or parasitic (Quénehervé, 2009). In practice, some of these antagonistic microorganisms are used in soil treatment and other as induction of plant defense/resistance/tolerance. In soil treatment, the parasitic fungus *Paecilomyces lilacinus* (Pl251) (Tandigan and Davide, 1986; Davide, 1988) was developed and formulated as water dispersal granules made of 10^{10} viable spores per g of the fungus. However, published data on its long-term efficacy on banana nematodes under field conditions are still lacking (Quénehervé, 2009). The trapping fungus *Arthrobotrys* sp. also demonstrated some efficacy under laboratory conditions (Lopez *et al.*, 2000).

Some bacteria were reported to reduce nematodes population such as a particular strain of *Bacillus thuringiensis* (Bt var. Kurstaki, strain LBT-3) (87 % reduction after two months treatments) and *Corynebacterium paurometabolum* (C-924) (85 % reduction) under field conditions (Fernandez *et al.,* 2005). As obligate parasites of plant-parasitic nematodes, *Pasteuria penetrans* was reported to suppress root-knot nematodes with its endospores that infect all development stages of the nematodes (Preston *et al.*, 2003).

AMF have also received increasing attention in the control of nematodes. This will be detailed in section 2.3 below.

2.3. Arbuscular mycorrhizal fungi

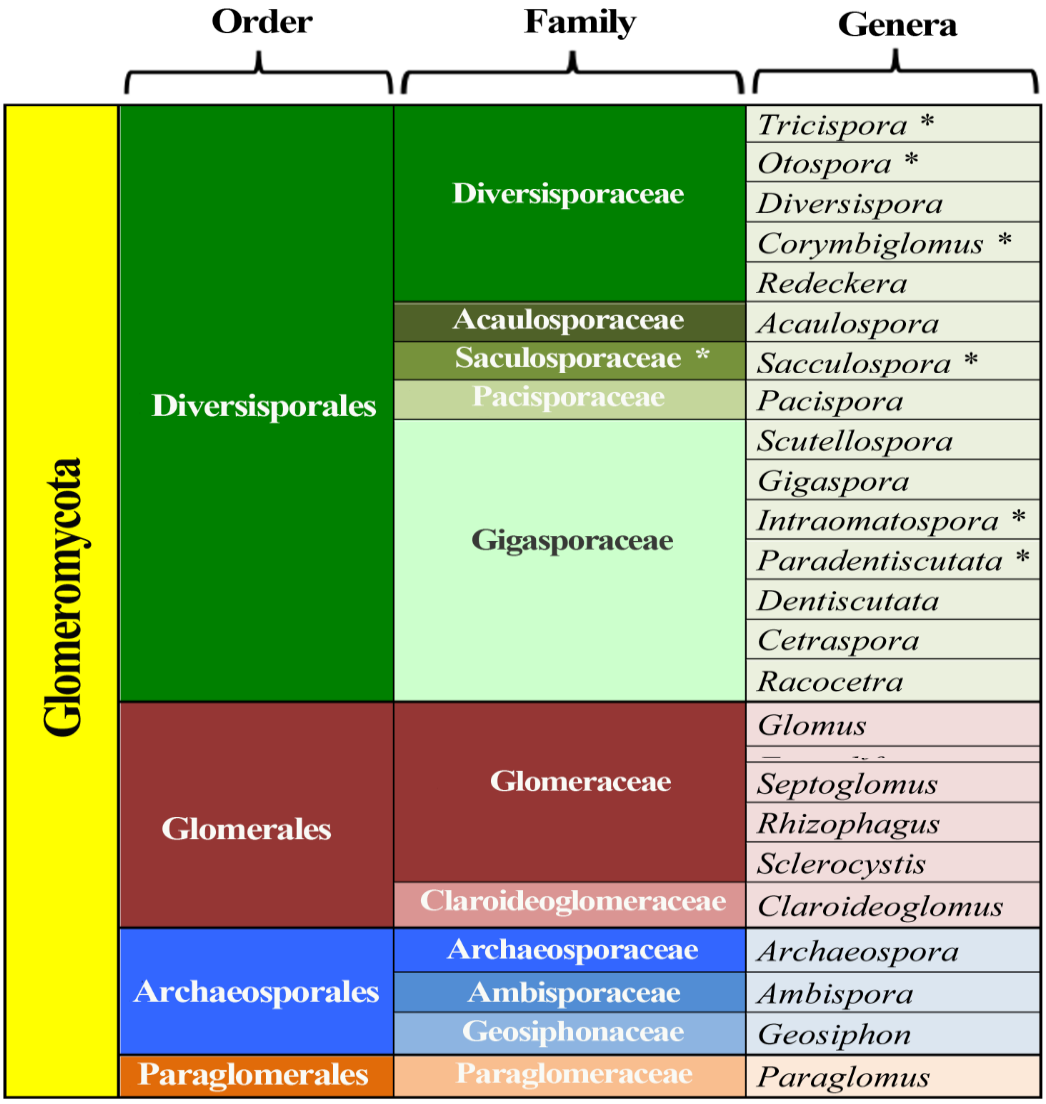
Mycorrhizas are mutualistic associations between soil fungi and roots (Frank, 1885; Boostani *et al.*, 2014). Several types of mycorrhizas exist, among which the most prevalent is the arbuscular mycorrhizas (Bever *et al.*, 2001; Garg and Chandel, 2010). AMF are key microorganisms within the rhizosphere, which form obligate symbiotic associations with nearly 80% of plant species (Barea *et al.*, 2002), including bananas (Whipps, 2004). AMF perform many critical functions: they increase plant nutrients and water uptake and thus yield, improve plant protection against abiotic/biotic stresses and improve soil structure (Whipps, 2004; Garg and Chandel, 2010).

Mycorrhizal colonization of roots is known to modify several aspects of plant physiology including mineral nutrients composition, hormonal balance, carbon allocation patterns, etc. (Harley and Smith 1983; Smith *et al.*, 1994; Azcón- Aguilar and Barea, 1996). Consequently, the mycorrhizal symbiotic status changes the chemical composition of root exudates, while the development of the mycelium in the soil serves as a carbon source to rhizosphere microbial communities and induce physical modifications (Barea *et al.*, 2002).

In the last two decades, more and more studies revealed that AMF are important biocontrol agents that are involved in plant defenses (Barea *et al.*, 2002; Koffi *et al.*, 2013a; Vos *et al.*, 2013). These aspects will be detailed in section 2.3.2 below.

2.3.1. AMF: systematic and biology

Arbuscular mycorrhizal fungi belong to the Glomeromycota phylum (Schüßler *et al.*, 2001) which contains 4 orders (diversisporales, glomerales, archaeosporales, paraglomerales) and 25 genera (Fig. 7) distributed in 11 families (Redecker *et al.*, 2013). These soil fungi are obligate biotrophs, unable to complete their life cycle in absence of a suitable host plant (Smith and Read, 2008).



	Order	Family	Genera
Glomeromycota	**Diversisporales**	**Diversisporaceae**	*Tricispora* *
			Otospora *
			Diversispora
			Corymbiglomus *
			Redeckera
		Acaulosporaceae	*Acaulospora*
		Saculosporaceae *	*Sacculospora* *
		Pacisporaceae	*Pacispora*
		Gigasporaceae	*Scutellospora*
			Gigaspora
			Intraomatospora *
			Paradentiscutata *
			Dentiscutata
			Cetraspora
			Racocetra
	Glomerales	**Glomeraceae**	*Glomus*
			[illegible]
			Septoglomus
			Rhizophagus
			Sclerocystis
		Claroideoglomeraceae	*Claroideoglomus*
	Archaeosporales	**Archaeosporaceae**	*Archaeospora*
		Ambisporaceae	*Ambispora*
		Geosiphonaceae	*Geosiphon*
	Paraglomerales	**Paraglomeraceae**	*Paraglomus*

Figure 7 : Arbuscular mycorrhizal fungi classification (Redecker* et al.*, 2013).
Asterisks indicate insufficient evidence in the consensus classification of the Glomeromycota, but no formal action taken.

The establishment of the AMF symbiosis starts with the colonization of a compatible root by hyphae produced by AMF propagules (e.g. asexual spores) (Requena *et al.*, 1996; Garg and Shandel, 2010). Following attachment to the root surface via an appressorium or hyphopodium (Garg and Shandel, 2010), fungal hyphae penetrates into the root via a pre-penetration apparatus, which guides the fungal hyphae through the root cells toward the cortex (Bücking *et al.*, 2012). In the cortex the hyphae enter the apoplast, and grow laterally along the root axis, and penetrate into inner root cortical cells, forming branches that by continuous dichotomous branching develop into characteristic highly branched arbuscules (Fig. 8) (Garg and Shandel, 2010; Bücking *et al.*, 2012). Arbuscules are specialized hyphae that form inside the root cells and are the principal site of mineral nutrients transfer to the plant and of carbon acquisition by the fungus (Requena *et al.*, 2007). Some fungi also form vesicles which are fungal storage organs in the root apoplast (Requena *et al.*, 2007; Garg and Shandel, 2010; Bücking *et al.*, 2012) and probably reproduction structures (Declerck et al., 1996). After host colonization, the fungal mycelium grows out of the root exploring the soil in search of mineral nutrients or colonizing other susceptible roots. Despite its coenocytic nature, the mycelium that is formed within the root, (i.e. the intraradical mycelium – IRM) differs morphologically and functionally from the mycelium that grows into the soil (i.e. the extraradical mycelium – ERM) (Garg and Shandel, 2010). The ERM absorbs nutrients from the soil and transfers these nutrients to the host root. The IRM on the other hand releases nutrients into the interfacial apoplast and exchanges them against carbon from the host. The fungus uses these carbon resources to maintain and to enlarge the ERM, for cell metabolism (e.g. active uptake processes, nitrogen assimilation), and for the development of spores, which are able to initiate the colonization of novel plants (Garg and Shandel, 2010; Bücking *et al.*, 2012). The fungal life cycle is completed after formation of asexual spores on the ERM (Requena *et al.*, 2007; Garg and Shandel, 2010).

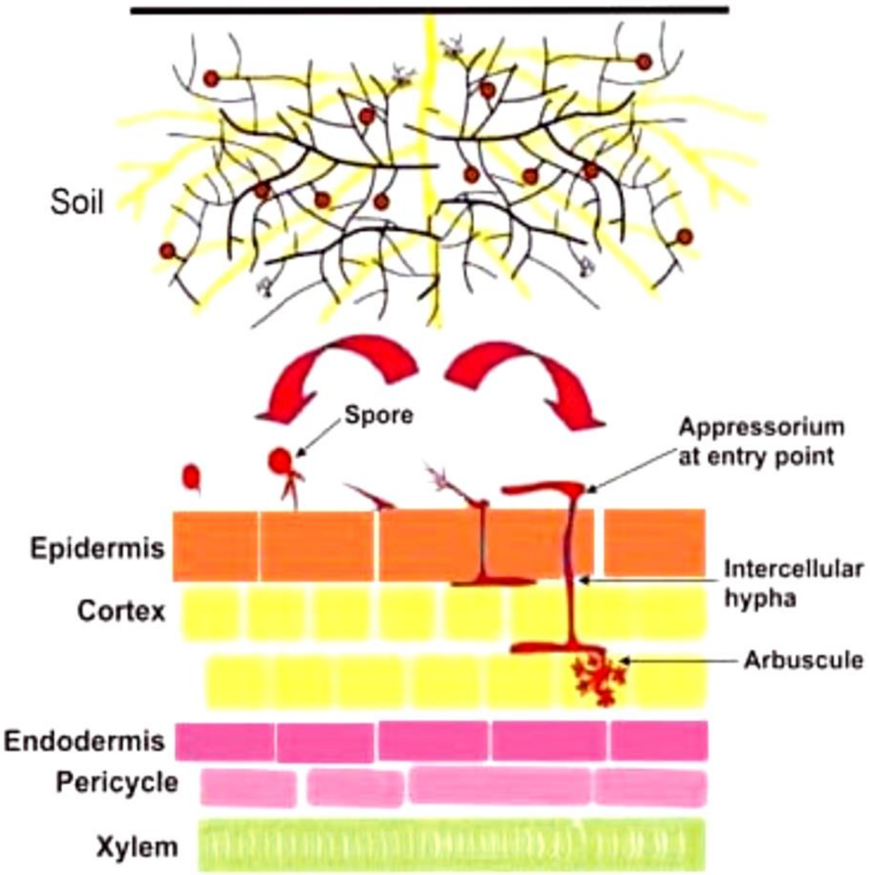


Figure 8: Life cycle of an AMF and the different steps during AMF development (Garg and Shandel, 2010)

2.3.2. AMF – Biocontrol of nematodes

Several studies have reported on the benefits of AMF to plants: increased nutrients uptake (Smith and Read, 2008; Boostani *et al.*, 2014), improved plant resistance/tolerance/tolerance to abiotic stresses (e.g. salinity, drought, pollutants… - see review of Plouznikoff *et al.*, 2016) and biotic stresses (Azcón-Aguilar and Barea, 1996).

In biotic stress management, the negative-antagonistic interaction of AMF with various plant pathogens is the reason for their use as biocontrol agents (Tahat *et al.*, 2010). AMF symbiosis has been shown to reduce the damage caused by soil-borne pathogens (Azcón-Aguilar and

Barea, 1996; Garg and Shandel, 2010; Tahat *et al.*, 2010). Consistent reduction of disease symptoms has been described for fungal or pseudo-fungal pathogens belonging to the genus *Phytophthora, Gaeumannomyces, Fusarium, Pythium, Rhizoctonia, Sclerotium, Verticillium, Aphanomyces* (Azcón-Aguilar and Barea, 1996), for nematodes such as *Rotylenchus, Pratylenchus* and *Meloidogyne* (Azcón-Aguilar and Barea, 1996; Schouteden *et al.*, 2015) and for bacteria such as *Ralstonia* (Yuan *et al.*, 2016). For instance, Cordier *et al.* (1996) reported that *P. nicotianae v. parasitica* proliferation was greatly reduced when tomato roots were colonized by AMF and *P. parasitica* compared to non-mycorrhizal tomato roots. Hussey and Rancadori (1978) found that colonized cotton by *G. margarita* was a less suitable host for *P. brachyurus* compared to the non-mycorrhizal plants. This was evidenced by the fewer number of nematodes enumerated in the cortical tissues intensively colonized by the AMF as compared to tissues lacking the symbiont. In the same way, tomato plants colonized by *G. fasciculatum* showed significantly lower numbers of giant cells produced by the root-knot nematode *M. incognita*, although roots did not prevent the penetration by the nematode juveniles (Mukerji and Ciancio, 2007). Yuan *et al.* (2016) recently reported that the application of AMF significantly reduced the abundance of *Ralstonia solanacearum* in tobacco roots.

The protective effects of AMF inoculation may be both systemic and localized, and there is evidence supporting both types of induced resistance/tolerance (Linderman, 1994). Different mechanisms were proposed for the biocontrol via AMF.

2.3.2.1. AMF mechanisms in pathogens biocontrol

Many mechanisms have been proposed to explain the greater resistance/tolerance of mycorrhizal plants to pests and diseases. These include improvement of plant nutrition, root damage compensation, competition for photosynthates or colonization/infection sites, anatomical or morphological changes in the root system, changes in the microbial populations developing in the ‘myco’-rhizosphere, and activation of plant defense mechanisms (Azcón-Aguilar and Barea, 1996; Garg and Shandel, 2010; Boostani *et al*., 2014).

Improved nutrient status of the host plant: The increasing uptake of nutrients induced by the AMF symbiosis result in more vigorous plants; thus the plant itself may be more resistant or tolerant to pathogen attack (Linderman, 1994; Azcón-Aguilar and Barea, 1996).

Damage compensation: Linderman (1994) reported that AMF increase host tolerance by compensating the loss of functional roots and biomass caused by soil-borne pathogens including fungi and nematodes (Cordier *et al.*, 1996). This represents an indirect contribution to the biological control through the conservation of root system function both by AMF hyphae growing out into the soil and increasing the root absorbing surface area as well as by the maintenance of root cell activity through arbuscules formation (Gianinazzi-Person *et al.*, 1995; Cordier *et al.*, 1996).

Competition for host photosynthates: AMF and root pathogens rely on host photosynthates for their development. They may thus compete for carbon compounds in the roots (Linderman, 1994; Smith and Read, 1997). When AMF have prior access to the photosynthates (i.e. in pre-mycorrhized plants), they may have a growth advantage over the pathogens (Linderman, 1994; Azcón-Aguilar and Barea, 1996).

Competition for infection/colonization sites: Dehne (1982) illustrated how fungal root pathogens and AMF, although colonizing the same host tissues, usually develop in different root cortical cells, indicating some sort of competition for space. Both localized and non-localized mechanisms may exist, probably depending on the pathogen (e.g. fungi, nematodes) (Azcón-Aguilar and Barea, 1996. Cordier *et al.* (1996) showed that *P. nicotianae v. parasitica* is reduced in AMF colonized regions of AMF root systems, and that the pathogen does not penetrate arbuscule-containing cells. This means that localized competition occurs, and that even in the absence of systemic resistance/tolerance, resistance/tolerance was still induced at some distance from the AMF colonized tissue (Azcón- Aguilar and Barea, 1996; Tahat *et al.*, 2010).

Morphological changes: induced bioprotection in the root system: It has been demonstrated that AMF colonization induces remarkable changes in root system morphology, as well as in the meristematic and nuclear activities of root cells (Atkinson *et al.*, 1994; Tahat *et al.*, 2008). Roots colonized by AMF are more highly branched compared to non-colonized plants and also the adventitious root diameters are larger (Berta *et al.*, 1993), which can provide more infection sites for a pathogen (Hooker *et al.*, 1994). They reported that the infection of tomato and cucumber by *Fusarium* wilt might slow down due to the morphological changes in the root cells of the endodermis of mycorrhized plants which include lignification. The raising lignification may protect the roots from penetration by other pathogens, while elevating the phenolic metabolism within the host plant (Tahat *et al.*, 2010). The colonization of tomato root by *G. mosseae* lead to a larger root size and more branching which increase the number of root tips, length, surface area and root volume (Tahat *et al.*, 2008). Root damage by *Gaeumannomyces graminis* var. *tritici* was systemically reduced when barley plants were highly colonized as compared to the non-mycorrhizal barley (Khaosaad *et al.*, 2007).

Microbial changes in the mycorrhizosphere: Changes in soil microorganism populations induced by AMF symbiosis may lead to stimulation of certain components of the microbiota, which in turn may be antagonistic to root pathogens (Azcón-Aguilar and Barea, 1996; Barea *et al.*,2002a). Meyer and Linderman (1986) reported that application of extracts of rhizosphere soil from mycorrhizal plants reduced the number of sporangia formation of *Phytophthora cinnamomi* in comparison with extracts of rhizosphere soil from non-mycorrhizal plants. Caron (1989) also reported a reduction in *Fusarium* species populations in the soil surrounding mycorrhizal tomato roots as compared with the soil of non-mycorrhizal controls.

AMF and PGPR (from Rhizobacteria) may cooperate in several ways, including their mutual establishment in the rhizosphere, in improvement of plant rooting (Azcón-Aguilar and Barea, 1996), enhancement of plant growth and nutrition, biological control of root pathogens, and improved nodulation in the case of legumes (Barea *et al.*, 1995). Furthermore, Varma (1999) reported that changes in root exudates affect the microbial communities around the roots leading to the formation of mycorrhizosphere (rhizosphere of the mycorrhizal plant). Barea *et al.*, (2002b) confirmed this observation and reported that the survival of *Azotobacter paspali* increased in the mycorrhizosphere.

Changes in chemical constituents of plant tissues (root exudates): Morandi (1996) reported that phytoalexins toxic components are not detected during the first stages of AMF colonization but can be detected in the later stages of symbiosis showing changes in chemical constituents due to AMF presence. Barea *et al.* (2002a) added that wall-bound peroxidase activity has been detected during the initial stage of AMF colonization and cortical cells containing AMF (*G. mosseae*) are immune to the pathogen and exhibit a localized resistance/tolerance response (Cordier *et al.*, 1996). Root exudates from mycorrhizal strawberry plants suppressed the sporulation of *Phytophthora fragariae* in *in vitro* study (Norman and Hooker, 2000). Some reports indicate that phosphorus-induced changes in root exudation could reduce the germinations of pathogen spores (Graham, 1982; Sharma *et al.*, 2007).

2.3.2.2. AMF biocontrol of nematodes

In recent years, research on the role of AMF in the control of nematode populations has received particular attention. Many studied have reported a decrease in population of nematodes in presence of AMF, under greenhouse (Elsen *et al.*, 2008; Dos Anjos *et al.*, 2010; Vos *et al.*, 2012, 2013) as well as under *in vitro* (Elsen *et al.*, 2001a, 2003; Koffi *et al.*, 2013a) culture conditions and several mechanisms (see section 2.3.2.1) have been proposed (see Fig. 9 – Schouteden *et al.*, 2015).

Direct competition: Competition between AMF and nematodes has been reported because both organisms colonize/infect the same root cells (Jung *et al.*, 2012). According to Azcón-Aguilar and Barea (1996), the biocontrol effect is mostly remarkable in plants colonized by AMF prior to any attack by a nematode. Dos Anjos *et al.* (2010) reported that the mycorrhization of sweet passion fruit's prior to *M. incognita* infestation reduced the reproduction of the nematodes whereas co-inoculation had no effect. Root colonization by *R. irregularis* in *in vitro* banana plantlets was not affected either by *R. similis* (Koffi *et al.*, 2013a) or by *P. coffeae* in transformed carrot roots (Elsen *et al.*, 2003).

Induced resistance/tolerance: Induced resistance/tolerance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (Choudhary *et al.*, 2007). Systemic induced resistance/tolerance (ISR) is a form of induced resistance/tolerance where plant defenses are preconditioned by prior infection or treatment that results in resistance/tolerance against subsequent challenge by a pathogen or parasite (Choudhary *et al.*, 2007). Interestingly, in AMF it was shown that root colonization of one part of the root system induced resistance/tolerance in the whole root system against nematodes (Elsen *et al.*, 2008). The root system of banana was divided over two physically separated compartments with one side pre-inoculated with AMF against *R. similis* and *P. coffeae*, while the other being free of any nematodes. They observed a significant reduction in the number of nematodes in both compartments. In a similar split-root system with tomato, Vos *et al.* (2012) observed that colonized roots (developing in one compartment) induced resistance/tolerance against *M. incognita* or *P. penetrans* in non-colonized roots developing in the other compartment.

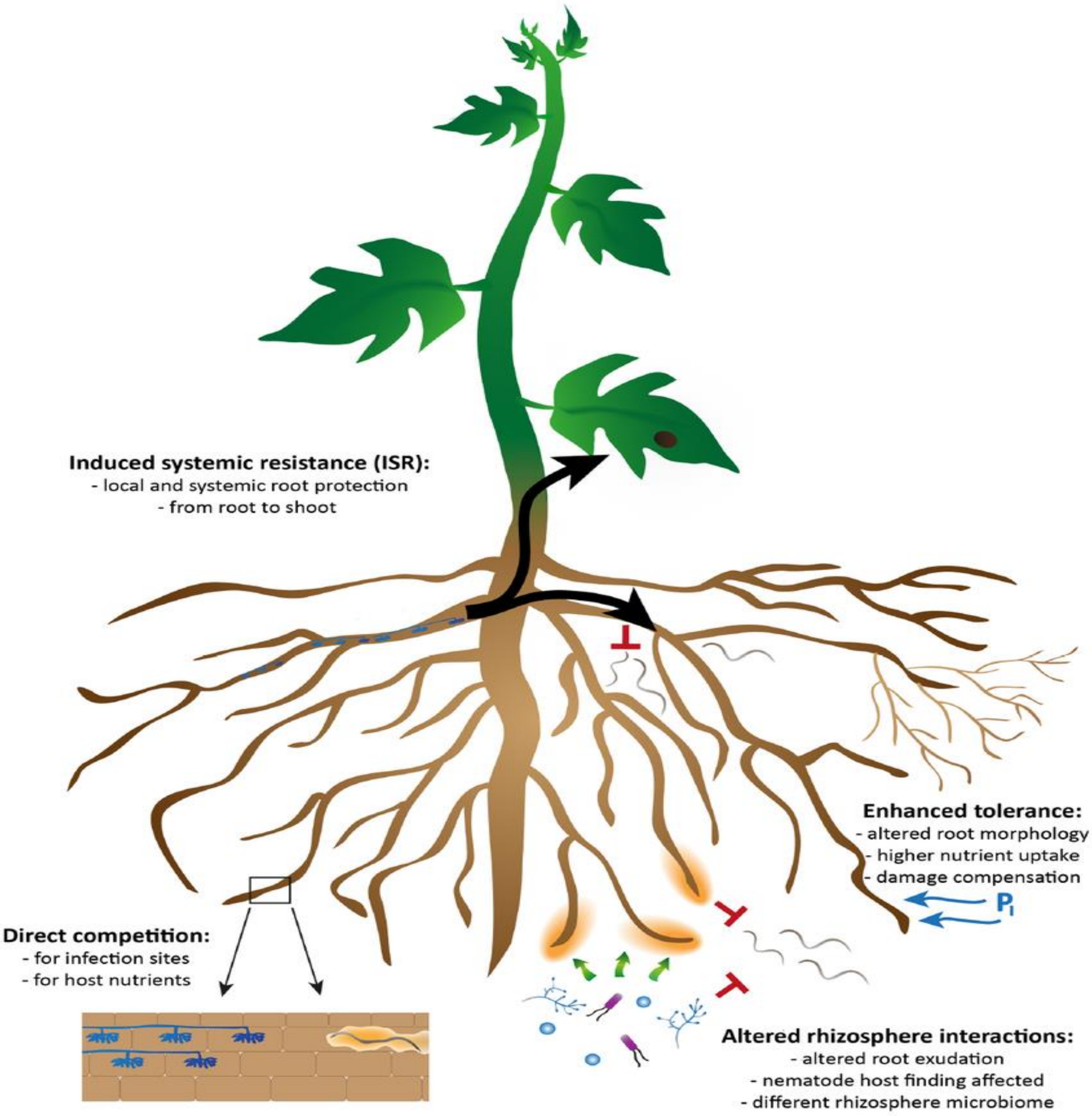


Figure 9: Overview of the possible mechanisms by which arbuscular mycorrhizal fungi can exert biocontrol against plant-parasitic nematodes (Schouteden *et al.*, 2015).

They consist of direct effects of AMF on the pathogen, involving competition for space and nutrients (bottom left) and indirect plant-mediated effects, involving damage compensation and enhanced tolerance (top right). The latter can be further divided into the effects of AMF on plant defense induction (ISR; top left) and altered rhizosphere interactions through changes in plant root exudation (bottom right).

2.4. *Crotalaria*

2.4.1. Taxonomy of *Crotalaria* species

Crotalaria is a leguminous woody shrub belonging to the order Fabales and family Fabaceae. The genus *Crotalaria* contains more than 500 species. They are mainly distributed in tropical and subtropical climate with the principal center of diversity in the central-south and eastern regions of Tropical Africa (Devecchi *et al.*, 2015). *Crotalaria* species are erected plants (Fig. 10a) which height varies from 1.5 m (C. *spectabilis*) up to 4 m (*C. juncea*) (Mosjidis and Wang, 2011; Dorel *et al.*, 2013). *Crotalaria* species have alternate simple leaves with dark yellow flowers (Fig. 10b) arranged in long indefinite loose inflorescences at the extremity of the stem. Its fruit is a pod containing dark brown seeds (Fig. 10c). The root system consists of a short pivot with many lateral roots which may include large amounts of active coral-shaped nodules (up to 2.5 centimeters in diameter) (Dorel *et al.*, 2013).

(a) Plant *Crotalaria*

(b)

(c)

Figure 10: Plant (a) of *Crotalaria spectabilis* and its flowers (b) (Maddox *et al.*, 2009) **and seeds (c)** (Anene, 2006)

2.4.2. Importance of *Crotalaria* species

Crotalaria is a fast growing crop, which species have numerous potentials for agriculture (Germani and Plenchette, 2004; Wang and McSorley, 2012). Many of the *Crotalaria* species (*C. juncea*, *C. spectabilis*, *C. pallida* …) are important sources of fiber, animal forage, or green manure and some have medicinal or ornamental value (Lewis *et al.*, 2005; Devecchi *et al.*, 2015). Some of the species such as *C. juncea* (sun hemp) are cultivated for industrial use. For example, in India and Pakistan, this plant is cultivated for the strong baste fiber extracted from the bark, which is more durable than jute (Duke, 1983; Wang and McSorley, 2012). Its fiber (one of the oldest known fibers in the Indo-Pakistan subcontinent) is used in twine, rug yarn, cigarette and tissue papers, fish-nets, sacking, canvas and cordage (Duke, 1983). However in tropical and subtropical areas, *Crotalaria* species are widely grown for soil health and nematodes controls.

2.4.2.1. *Crotalaria* species in soil quality

Crotalaria is a leguminous plant and is thus able to fix atmospheric nitrogen via its symbiosis with rhizobium (Wang *et al.*, 2002; Dorel *et al.*, 2013). The plants are tilled into the soil two months after planting to increase the soil organic matter and therefore to increase nitrogen content (Wang *et al.*, 2002). Therefore, *Crotalaria* species are used as soil amendment and cover crop. In soil amendment, *Crotalaria* species are commonly used as green manure and as organic mulch (cover crop) (Wang and McSorley, 2012). As green manure the crop is grown for 2 to 3 months before cash crop planting and then incorporated into the soil at early blooming stage whereas as organic mulch, the cover crop is mowed and left on the soil surface (Wang and McSorley, 2012). Fischler *et al.* (1999) reported that when *Crotalaria* was early sown in the season and applied as mulch before sowing maize, the maize grain yield was increased by 68%.

In soil restoration, *Crotalaria* (tolerant to drought) is cultivated as cover crop (Duke, 1983) during fallow. In fallow, the presence of a pivot root associated with a network of dense lateral roots favor the filtration and infiltration capacities in the upper horizons of the soil profile (Dorel *et al.*, 2013) improving the drainage of the soil. Their fast growing capacity confers them the ability to overhanging weeds canopy (Wang *et al.*, 2002; Wang and McSorley, 2012).

2.4.2.2.*Crotalaria* species in nematode control

Several studies have reported on the use of *Crotalaria* species in nematodes management (Wang *et al.*, 2002; Germani and Plenchette, 2004; Risède *et al.*; 2010a; Wang and McSorley, 2012). The plants produce allelochemicals that are toxic or inhibitory to some pests and provide niches for antagonistic flora and fauna trapping nematodes (see chapter 2). Indeed, several papers have reported that *Crotalaria* species are non-host for root-knot nematodes (*Meloidogyne spp*.) and poor-hosts for some migratory nematodes such as *R. similis* (nematodes fail to reproduce in their roots) (Wang *et al.*, 2002; Thoden *et al.*, 2009). Kushida *et al.* (2003) cultivated soybean and *C. juncea* or *C. spectabilis* in presence of the soybean cyst nematode *Heterodera lycines*. They observed that the number of second-stage juveniles of *H. glycines* entering *Crotalaria* roots (*C. juncea* and *C. spectabilis*) was similar to the number entering soybean roots, but most of the nematodes in *C. juncea* and *C. spectabilis* roots did not develop to an advanced stage of growth. Germani and Plenchette (2004) also reported that inoculated juveniles (J2) of the root-knot nematodes *M. javanica* and *M. incognita* developed into adult females in presence of tomato, while in presence of several *Crotalaria* species they rarely developed beyond J3 stage. This confirmed that *Crotalaria* species are non-hosts or poor hosts for these nematodes. For instance, Jasy and Koshy (1994) demonstrated that leaf extracts of *C. juncea* were lethal to *R. similis* at dilutions of 1:5 within 24 hours. Wang and McSorley (2012) also reported that some *Crotalaria* species are efficient in suppressing migratory nematodes.

Wang *et al.* (2002) reported different efficiencies of *Crotalaria* species to *Pratylenchus* and *Helicotylenchus spp*. For instance, *P. zea* can penetrate roots of *C. breviflora*, *C. spectabilis*, *C. retusa*, and *C. juncea*, and *P. brachyurus* roots of *C. juncea* and *C. spectabilis.*

Crotalaria spectabilis (also named Showy rattlebox and sometimes showy crotalaria - Maddox *et al.*, 2009), is grown as green manure and for stabilizing the soil (Fem *et al.*, 2014). Showy rattlebox has simple, alternate leaves, with persistent bract and yellow flowers and can grow in rustic environment (Maddox *et al.*, 2009).

The seeds and other above-ground parts of showy crotalaria contain the pyrrolizidine alkaloid monocrotaline (MCT), which is toxic to root-knot nematodes (*M. spp.)* (Maddox *et al.*, 2009; Fem *et al.*, 2014). The plant growth rate and effective control of root-knot and sting nematodes make it a very useful green manure and biocontrol crop (Fem *et al.*, 2014).

III. RESEARCH OBJECTIVES

III. RESEARCH OBJECTIVES

Bananas are produced in nearly 140 countries and territories across the tropics and subtropics. Their production is mostly for home consumption and local markets, while only 15% is intended for export (Promusa, 2016). These bananas for international trade are among the most pesticide-treated crops. Indeed, they are threatened by numerous pests and diseases, requiring control measures often based on the repeated applications of high doses of various pesticides.

One of the most problematic biotic constraint in banana plantations is the burrowing nematode *R. similis* (Quénehervé, 2009). This nematode was reported to decrease yield by up to 75% in the worst cases (Quénehervé, 2009). Until the 19th century its control was most often based on chemicals (Risède *et al.*, 2010b). However, these molecules may have serious side effects on human health and environment, thus requiring the development of alternative control measures. Among these are the use of biological control agents such as AMF (Sikora and Pocasangre, 2005; Quénehervé, 2009). Several studies have reported the effectiveness of these obligate root symbionts for the control of nematodes (Elsen *et al.*, 2001a, 2003, 2008; Dos Anjos *et al.*, 2010; Vos *et al.*, 2012, 2013; Koffi *et al.*, 2013a).

Nowadays, most studies on the role of AMF in the control of nematodes were conducted in pots or with transformed roots organs (e.g. carrot roots). Both systems have yield critical information on the mechanisms of bio-protection (Schouteden *et al.*, 2015) but have also shown a number of limitations. These are, in particular, the risk of interferences with unwanted microbial contaminants in pot cultures and the use of excised transgenic roots (i.e. in the absence of photosynthetic tissues), and therefore the absence of a true C-source, for the root organ cultures (Fortin *et al.*, 2002). One option to tackle these problems was proposed by Koffi *et al.* (2009). These authors developed an *in vitro* cultivation system associating autotrophic micropropagated banana plantlets with AMF, facilitating the tripartite interaction study between bananas, AMF and nematodes.

Biocontrol of nematodes with push pull plants (e.g. *C. spectabilis*) is also well documented (Good *et al*, 1965; Wang and McSorley, 2012). *The push pull strategy for controlling agricultural pests consist in using repellent "push" plants and trap "pull" plants* (Cook *et al.*, 2007).Interestingly, *C. spectabilis* is a highly mycotrophic plant (Germani and Plenchette, 2004). Therefore its combination with AMF to control nematodes may represent an interesting novel avenue for the integrated management of these pests.

Here, we aimed to investigate the effects of the AMF *R. irregularis* MUCL 41833 alone or in combination with *C. spectabilis*, on the burrowing nematode *R. similis* (infestation and population within roots) either under *in vitro* culture conditions or within pots. Three sub-objectives were addressed.

(1) Investigate the effects of AMF on *R. similis* populations and root infestation on banana plantlets grown under autotrophic *in vitro* culture conditions. Indeed, so far, most studies were conducted in pots (Vos *et al.*, 2012) or *in vitro* on excised transformed roots (Elsen *et al.*, 2001a, 2003). In pots, the presence of unwanted microorganisms, and thus interferences with the effects of the AMF, cannot be excluded. *In vitro*, there is the lack of a true sink for C-minerals exchange between the plant and the fungus and the medium contains sugars, which may impact the tripartite interaction (Fortin *et al.*, 2002). Koffi *et al.* (2013a) investigated for the first time the effects of *R. irregularis* MUCL 41833 on the reproduction of *R. similis* in the susceptible banana cultivar (Grande naine) grown under *in vitro* culture conditions. A drastic decrease in symptoms as well as nematodes densities in roots was noticed. Using the same *in vitro* culture system, it was our objective to investigate whether the partially resistant cultivar (Yangambi km5) would yield similar results or would benefit more or less from the AMF than the highly susceptible cultivar Grande naine.

Associating AMF to a push pull plant is another option, never tested so far, to control the burrowing nematode *R. similis. The push pull strategy is a stimulo-deterrent diversion technique which consists in repelling the pest from the main crop using repellent (push) crops and attracting it to border of the field using trap (pull) plants* (Cook et al., 2007 ; Ratnadass *et al*., 2012). We hypothesized that this strategy can be extended to nematodes where AMF associated to banana will protect banana (push) and lured by the companion plants (*C. spectabilis*). This led to the second objective.

(2) Investigate the impact of AMF and *C. spectabilis* together on the control of *R. similis* in banana. It is well-known that AMF decreases nematodes populations in roots and that *C. spectabilis* suppress root-knot nematodes and decrease migratory nematodes populations in bananas (Wang *et al.*, 2002). However, the combined effects of AMF and *C. spectabilis* on *R. similis* has never been studies, to the best of our knowledge. Here we used a bi-compartmented pot system in which the roots of both plants (the banana and *C. spectabilis*) were physically separated but connected by the extraradical mycelium of the AMF. Root colonization by the AMF as well nematode population and root necrosis were evaluated.

Associating AMF to *C. spectabilis* may possibly induce changes in root exudation. Indeed, it is well-known that AMF modify the root physiology and thus the quantitative and qualitative exudates, further impacting the microbes developing in the vicinity of AMF-colonized roots (Tahat *et al.*, 2008, 2010). It is thus not excluded that AMF may similarly impact the exudates of *C. spectabilis* and possibly modify its effects on the nematode. This led to the third objective.

(3) Investigate the impact of roots exudates of AMF-colonized *C. spectabilis* on the movement and viability of *R. similis*. It is well known from the literature that some

molecules (for example pyrrolizidine alkaloid - monocrotaline, the main active secondary metabolite of *C. spectabilis*) impact nematodes reproduction (nematicidal effects) and mobility (repellent effects) (Thoden *et al.*, 2009; Marahatta *et al.*, 2012). However it is unknown whether the exudates of AMF-colonized *C. spectabilis* plants are similarly effective on the nematodes. Here, root exudates of *C. spectabilis* associated or not to *R. irregularis* and of banana plantlets were tested on the chemotaxis and viability of nematodes.

IV. MATERIALS AND METHODS

IV. MATERIALS AND METHODS

4.1. Biological material

4.1.1. Plant material

- **Banana (*Musa acuminata*):** two banana cultivars were used throughout the research thesis.
    - Yangambi km5 (AAA genome, Ibota group), provided by the International *Musa* Germplasm collection at the INIBAP Transit Centre (ITC, K.U. Leuven, Belgium). Yangambi km5 is a cultivar partially resistant to some nematodes (*Radopholus similis, Pratylenchus goodeyi* and *Pratylenchus coffeae*) (Viane *et al.*, 2003).
    - Grande Naine, clone CV902 (AAA genome, Cavendish group), provided by VITROPIC SA (Montpellier, France). Grande Naine is a cultivar particularly susceptible to *R. similis* (Wuyts *et al.*, 2007; Quénehervé *et al.*, 2008).

Both cultivars were grown *in vitro*. The plant material was proliferated, regenerated and rooted on the Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 30 g l^{-1} sucrose and 2 g l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA). For the proliferating medium, 10^{-3} M indole-3-acetic acid (IAA) and 10^{-2} M 6-benzilaminopurine (BAP) were added to the MS medium. The pH was adjusted between 6.12 and 6.15 before sterilization (Banerjee & De Langhe, 1985). Individual aseptic shoots (± 1 cm height) were inserted in the test tubes after sterilization and cooling of the medium (Fig.11). For regeneration and rooting, no plant growth regulators were added, but 0.5 g.l^{-1} of active charcoal. Eight aseptic shoots (± 3 cm height) were placed in sterile culture boxes (145 x 100 x 85 mm (Greiner Bio-one)). The plantlets were grown in a grow chamber at 27/25°C (day/night), with a relative humidity (RH) of 70%, a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}.

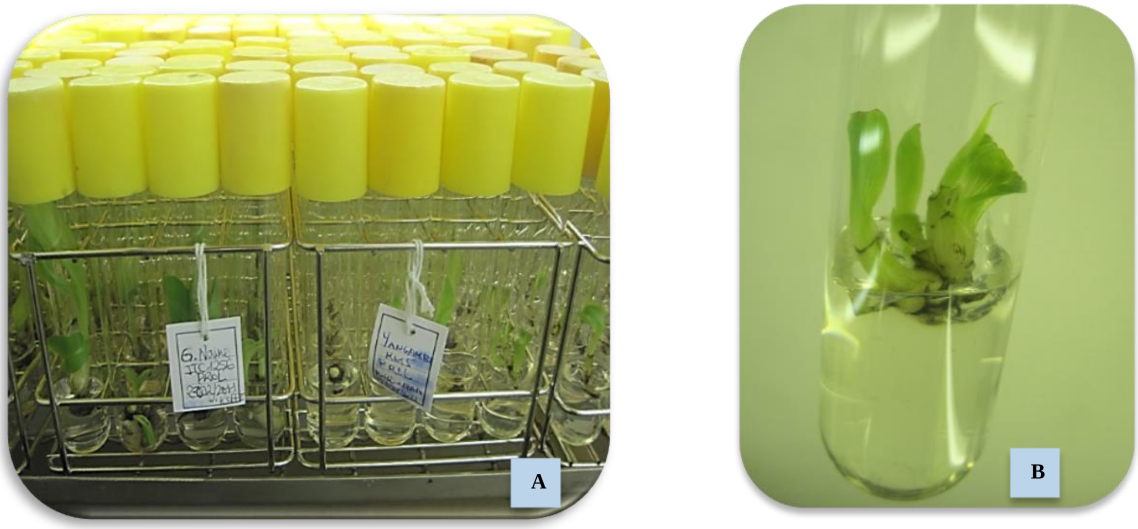


Figure 11 Banana shoots under *in vitro* culture conditions (proliferating medium)

A: Tubes containing 2 weeks-old shoots of banana c.v. Grande naine and Yangambi km5 in the proliferation phase.

B: Tube containing 1 week-old shoot of Yangambi km5 in the regeneration phase.

- ***Crotalaria spectabilis*** **(**Roth**):** It is a woody cover crop often named showy rattlebox. It produces allelochemicals (especially monocrotaline) that are toxic or inhibitory to some nematodes and provides niches for antagonistic flora and fauna trapping nematodes (Wang *et al.*, 2002). *Crotalaria* species are non-host for root-rot nematodes (*Meloidogyne spp*.) and poor-hosts for some migratory nematodes such as *R. similis* (nematodes fail to reproduce in their roots) (Wang *et al.*, 2002; Thoden *et al.*, 2009). The seeds of *C. spectabilis* were provided by Wolfseeds (Brazil) and used for *in vitro* and greenhouse studies.
    - For the *in vitro* studies, the seeds of *C. spectabilis* were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min, rinsed in sterilized

(121 °C for 15 min) deionized water and further germinated in 90 mm Petri plates filled with 35 mL of the Modified Strullu-Romand (MSR) medium (Declerck *et al.,* 1998) solidified with 3 g.l^{-1} Phytagel. The Petri plates were incubated in the dark at 27°C for 7 days before exposure to light for 2 days until use.

- For the greenhouse studies, the seeds were sown in 1 l pots containing 500 g of 1-2 mm diameter sterilized (2 x 8 hours at 110°C) sand (Euroquartz, Belgium) and maintained in a growth chamber at 28/24°C (day/night) with a RH of 80% during the first 2 weeks and 70% thereafter with a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 300 µmol m^{-2}s^{-1}. The pots were watered with deionized water when necessary. The plantlets were kept in the pots for 3 weeks reaching a size of 10 cm at the start of the experiment.

- **Medicago *truncatula* Gaertn:** It is a small annual leguminous plant. This plants is highly mycotrophic and often used as model plant for *in vitro* studies. It was used in our study as Mycelium Donor Plants (MDP – see section 4.2. below) (Voets *et al.*, 2005).

The seeds of the strain A17 were supplied by SARDI Genetic Resource Centre (Australia). They were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min, rinsed in sterilized (121 °C for 15 min) deionized water and further germinated in 90 mm Petri plates filled with 35 ml of the MSR medium solidified with 3 g.l^{-1} Phytagel. The Petri plates were incubated in the dark at 27°C for 4 days before exposure to light for 2 days until use.

4.1.2. Microbial material

- ***Rhizophagus irregularis*** (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler **MUCL 41833:** This AMF was formerly named *Glomus intraradices* Schenck & Smith. It was isolated from a soil of the Canary Islands (Spain). The strain was supplied by GINCO (http://www.mycorrhiza.be/ginco-bel/index.php) on Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 grown on the MSR medium. The strain was sub-cultured and maintained by associating isolated spores and colonized root pieces to *M. truncatula* roots in the MDP *in vitro* cultured system (see section 4.2. *in vitro* culture systems).

- ***Bradirhizobium arachidis*** (Wang, Chang, Zheng, Zhang, Zhang, Sui, Wang, Hu, Zhang and Chen) LMG 26795**:** The strain was supplied by BCCM/LMG (http://bccm.belspo.be/about/lmg.php). It was used to initiate the nodulation of *C. spectabilis* in pots as the culture substrate was sterilized sand (2 x 8 hours at 110°C) (1-2 mm diameter; Euroquartz, Belgium).

The bacteria was cultured in 90 mm Petri plates on Yeast Malt Agar (YMA). A subsample was then isolated from a one week-old culture and poured in a flask containing 100 ml liquid Difco^{TM} Yeast Malt (YM) Broth medium. The flask was maintained under agitation at 80 rpm during 10 days, at 28°C in the dark for inoculum production.

- ***Radopholus similis* (Cobb) Thorne:** the strain was isolated from banana roots in Uganda and provided by the Nematology laboratory of K.U. Leuven (Belgium) for *in vitro* and greenhouse studies.
    - For the *in vitro* studies, the nematodes (juveniles and adults) were maintained and sub-cultured under aseptic conditions on alfalfa (*Medicago sativa*) callus grow on the modified White’s medium (Elsen *et al.*, 2001b).
    - For the greenhouse studies, the nematodes were maintained and sub-cultured on disinfected carrot discs (Pinochet *et al.*; 1995), at 27°C in the dark.

4.2. The culture systems

4.2.1. *In vitro* culture systems

The AMF association to banana plantlets could be achieved either *in vivo* in pot cultures or *in vitro* in Petri plates. In pots, the method consist in adding AMF propagules to the culture substrate followed by planting the plantlet on the substrate. With this method, it takes 6 to 8 weeks for root colonization (Elsen *et al.*, 2005; Van der Veken *et al.*, 2008). *In vitro*, the method consist in introducing the banana plantlets in a network of AMF mycelium which is set up from a donor plant. With this method, it takes one week for root colonization (Voets *et al.*, 2009). Several growing methods were used throughout the thesis and are detailed below:

- **Mycelium Donor Plant (MDP) *in vitro* culture system**: The MDP *in vitro* culture system was developed by Voets *et al.* (2005) for the fast and homogenous mycorrhization of plantlets in actively growing AMF networks. This system was later adapted by Koffi *et al.* (2009) for banana plantlets and used in our study. Briefly, bi-compartmented Petri plates were constructed by introducing the base of a small Petri plate (55 mm diam. – named root compartment (RC)) in the base of a large Petri plate (145 mm diam. – named hyphal compartment (HC)). The two compartments were filled with MSR medium (15 ml and 100 ml in the RC and HC, respectively) without sucrose and vitamins, supplemented with 10 ml MES buffer (MSR_{SS} + MES) and solidified with 10 $g.l^{-1}$ Phytagel. The RC was leaned to the HC and a hole of ± 2 mm diameter was made in the base of both compartments. A 4 days-old *in vitro* produced seedling of *M. truncatula* was inserted in the system with the roots plated on the MSR_{SS} + MES medium in the RC and the shoot extending outside the bi-compartmented Petri plates via the hole (Fig. 12). A plug of medium containing ±100 spores of the AMF was inoculated in the vicinity of the roots. The holes were plastered with silicon grease (VWR International, Belgium) and the bi-compartmented Petri plates subsequently sealed with

Parafilm (Pechiney, Menasha, WI, USA). The Petri plates were placed in an incubator (18/21°C, a photoperiod of 16 h day^{-1}, a RH of 70% and a PPF of 300 µmol m^{-2}s^{-1}). Starting from the third week, the RC received weekly 10 ml of MSR$_{SS}$ + MES.

- **Banana plantlets mycorrhization in the AMF mycelium network**: After 8 weeks of incubation, the mycelium of the AMF crossed the plastic wall separating the RC from the HC and developed profusely in the HC, covering 80% of the surface within 4 weeks. At that time, four banana plantlets (3 weeks old) were introduced in the HC with their roots plated on the MSRss+MES medium in direct contact with the extraradical mycelium. To accommodate the plantlets to the system, the lid of the large Petri plate was replaced by another lid in which a 85 mm diam. hole was made. The hole was surmounted by a plastic bag with filter (0.22 µm over a length of 40 cm and a width of 0.5 cm COMBINESS, Belgium) (Fig 13). This compartment was named the shoot compartment (SC) and allowed gas exchanges. The plastic bag was glued to the lid of the Petri plate with hot melt glue (Metabo®). This cover was sterilized at 25 kGy (STERIGENIC, Fleurus, Belgium – Fig 13). The systems were placed in the growth chamber (25/27°C, a RH of 70% with a photoperiod of 16 h day^{-1} and a PPF of 300 µmol m^{-2} s^{-1}.

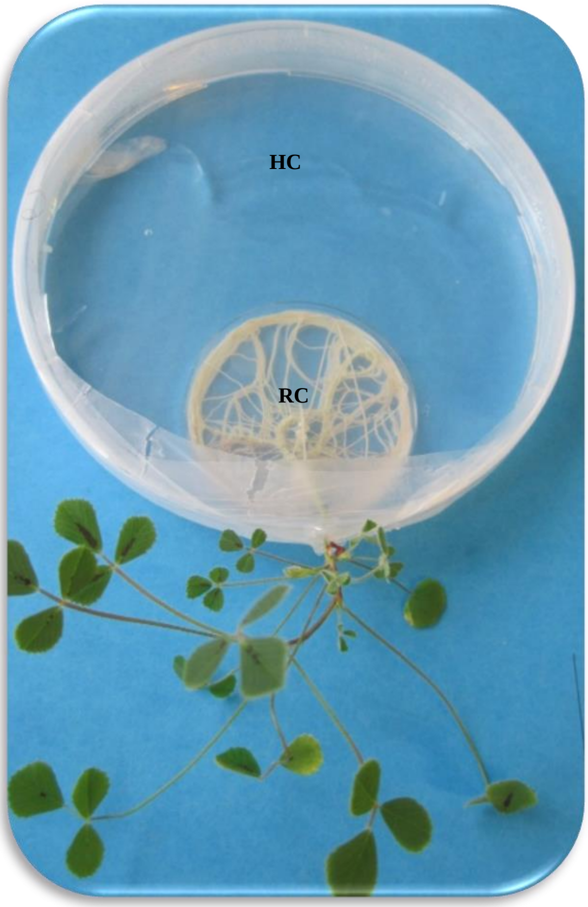


Figure 12 : Mycelium Donor Plant (MDP) *in vitro* culture system for the production of AMF. RC: root compartment; HC: hyphal compartment.

Figure 13 : Banana plantlets in the HC of the MDP *in vitro* culture system for the fast and homogenous mycorrhization

- **Set up of the half-closed arbuscular mycorrhizal plant (H-AMP) system for bananas:** After three weeks, the banana plantlets were removed from the AMF mycelium network and individually transferred in large Petri plate (145 mm diam.) filed with 100 ml of MSR_{SS} + MES medium solidified with 3 $g.l^{-1}$ Phytagel. Each plantlet was positioned vertically in the Petri plate, with the roots delicately placed on the medium and the shoot in upright position. A hole of 12 mm was made at one side of the lid to allow the shoot to extend outside the box (fig. 14). This system is called Half-closed arbuscular mycorrhizal plant (H-AMP) *in vitro* culture system. It was developed by Voets *et al.* (2005) and adapted later to banana by Koffi *et al.*, 2009). The hole was cautiously plastered with sterile Sealer (BREATHsealTM, Greiner Bio-one). The Petri plates were then sealed with Parafilm, covered with aluminium foil and incubated in a growth chamber set at 27/25°C (day/night) with a RH of 70% with a photoperiod of 16 h day^{-1} and a PPF of 300 µmol m^{-2} s^{-1}. Every week, the system received 50 ml of MSR$_{SS}$ +MES medium solidified with 3 g l^{-1} Phytagel.

- **Hydroponic *in vitro* system**: In order to facilitate the collection of roots exudates, the plants colonized or not by the AMF were grown in liquid MSR$_{SS}$ + MES medium using the H-AMP *in vitro* culture system as described above. The liquid MSR medium was provided via a hole of 14 mm diam. made on the lid of the Petri plate and surmounted with a half Falcon tube glued with hot melt glue (Metabo®) (Fig. 15).

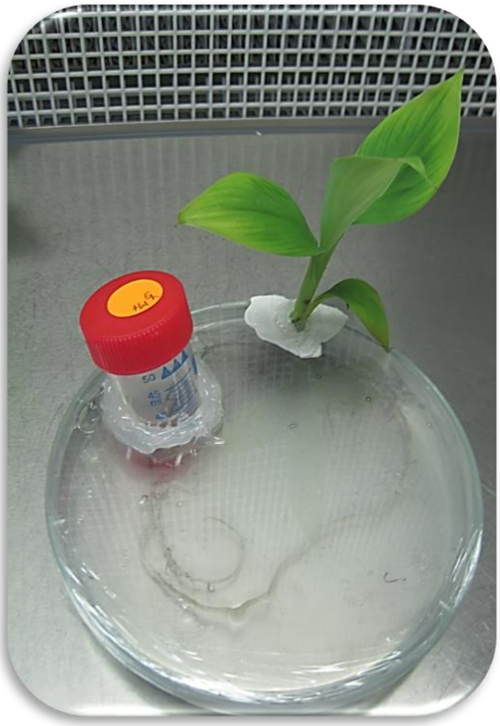


Figure 14 : Mycorrhizal banana plantlet in individual system (H-AMP *in vitro* culture system)

Figure 15 : AMF-colonized *C. spectabilis* plantlets in the H-AMP *in vitro* hydroponic culture system

4.2.2. Greenhouse systems: bi-compartmented pots

- **Compartmented Microcosm system for banana-AMF-*C. spectabilis* interactions in presence of *R. similis*.**

The microcosms consisted of pairs of plants (banana and *C. spectabilis*) that were set up in 3 l pots divided above-ground by a fixed talpa net (17x17 mm, Howitec Talpa Net, Nederland) to separate shoots and leaves of the two plants (Fig. 17). The pots were further divided below-ground in two compartments to avoid roots of both plants to intermingle (Fig. 17). In the first compartment, representing 1/3 of the pot volume, a pocket of nylon mesh (30 µm) was buried in the pot and filled with 0.8 kg sand (1-2 mm diameter; Euroquartz, Belgium), while the second compartment was filled with 1.6 kg sand (Fig. 17). The 1st and 2nd compartments were used to grow *C. spectabilis* and banana, respectively (Fig. 18). The plantlets received weekly 50 ml of Long Ashton (Hewitt, 1966). The banana plantlets were maintained in a growth chamber at 28/24°C (day/night) with a RH of 80% during the first 2 weeks and 70% thereafter with a photoperiod of 16 h day^{-1} and a PPF of 300 µmol m^{-2} s^{-1}. The pots were watered every 2 days with approximatively 150 ml of deionized water.

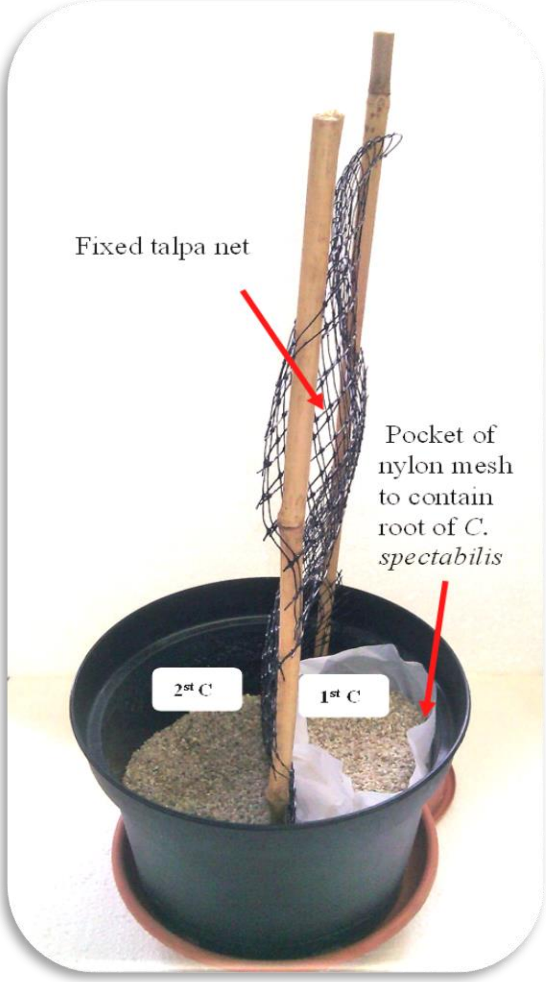


Figure 16: Microcosm pot design

Above ground part of the pot is divided in two with fixed talpa net to separate shoots of the two plants; below-ground the pot is divided in two compartments to avoid roots of both plants to intermingle; 1st C for *C. spectabilis* roots and 2nd C for banana

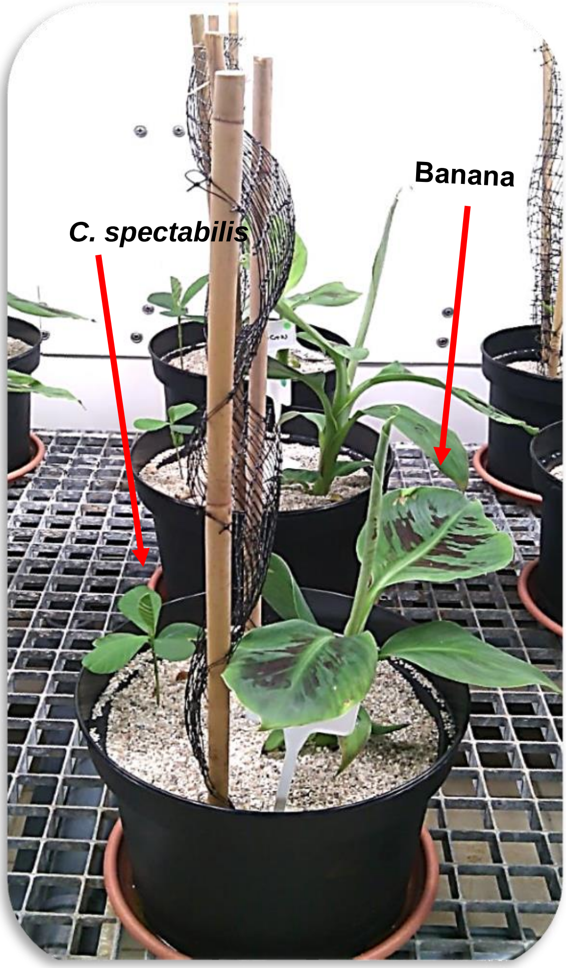


Figure 17: Microcosm system set with banana and *C. spectabilis*. The two plants are separated above-ground with fixed talpa net

4.3. Roots exudates collection protocol

Roots exudates were collected following a procedure adapted from the method developed by Tran *et al.* (2004). After culture in the liquid MSRss medium, the plantlets were removed and their roots gently washed with deionized sterilized water. The plantlets of *C. spectabilis* and banana were then placed in a sterilized Erlenmeyer flask containing 50 mL of sterilized (121°C for 15 min) deionized water and maintained at 45°C for 8 h and then at 28°C for 16 h. Only the roots were in contact with the water (Fig. 16). After this period, the solution was collected and filtered with acrodisc (2 µm) and further freeze-dried. The dried material was stored at -20°C until use.

Figure 18: Roots exudates collection process

Plants of *C. spectabilis* (A) and banana (B) in Erlenmeyer flask containing sterilized deionized water are maintained at 45°C in a water bath (C) for 8 h and then at 28°C for 16 h to stimulate roots exudation.

4.4. Parameters

- **Root colonization**

Roots colonization was evaluated after staining with ink (Parker®) and vinegar (Vierheilig *et al.,* 1998). Roots were first cleared 45 minutes in KOH (10%) and 30 minutes in H_2O_2 (3.5% for banana roots) at 70°C and rinsed several times with tap water. The cleared roots were subsequently stained 30 minutes with 5% ink-vinegar solution with white household vinegar (5% acetic acid). Root colonization was assessed by the method of McGonigle *et al.* (1990) (Fig. 19). 150 intersections were observed under a compound microscope (Olympus BH2, Olympus Optical, and GmbH, Germany) at 20-40× magnifications. Total root colonization (% RC), abundance of arbuscules (% A) and intraradical spores/vesicles (% V) were determined.

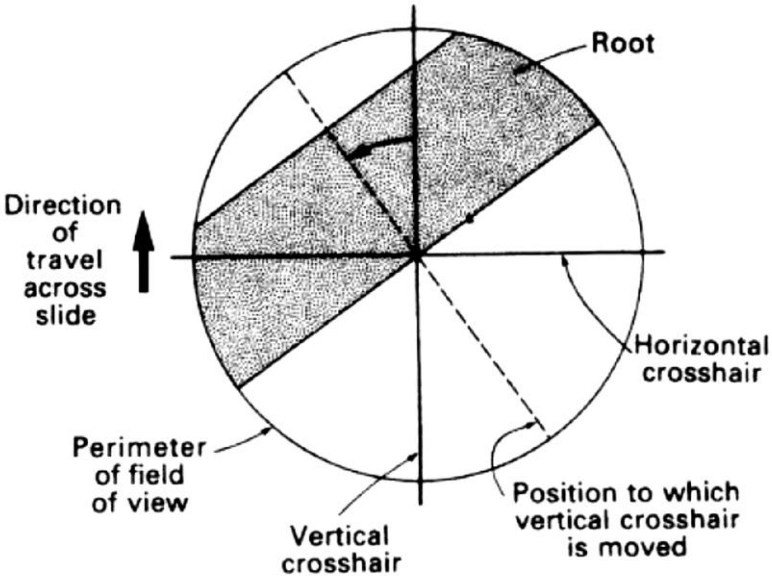


Figure 19 : A diagram to show how a magnified intersection perpendicular to the long axis of the root can be made when the root is aligned with its long axis at an angle to the vertical crosshair.
The stage is moved until the center of the crosshairs is contiguous with the first edge of the root reached. To make the perpendicular intersection, the vertical crosshair is then rotated as shown (From McGonigle *et al.* (1990) in New Phytologist).

- **Nematode population**

Nematodes and their eggs were collected from the medium using the modified Bearmann funnel (Coyne *et al.*, 2010) with a sieve of 5 µm to collect the aliquot. The modified Bearmann funnel consists of laying out tissue paper on a plastic sieve and placed in a plastic plate. The medium containing decayed roots was chopped in a small pieces and laid on the tissue paper. Then the medium was regularly humidified with distilled water when needed up to 3 days. Concerning nematodes in roots, 25 g of fresh roots of infested banana (fig. 20) and *C. spectabilis* plants were cut in 1 cm pieces and macerated in water with a blender for 30 sec. with three bursts of 10 sec. intervals. The nematodes were separated from the root tissues and debris using a series of sieves (250-100-25 µm). They were further collected from the 25 µm sieve with distilled water. The nematode solution was diluted with distilled water in a beaker of 200 ml. After homogenizing the solution, 3 x 2 ml were collected and nematodes counted with counting slide under binocular microscope. The number of juveniles, females, males and total number of nematodes per plant and the reproduction ratio (final population/initial population) were determined according to Speijer and De Waele (1997) (see below).

- **Root necrosis index (RNI)**

Root necrosis (Fig. 20) index (RNI) is the percentage of root surface showing necrosis. The RNI was evaluated on 10 functional roots randomly selected per plant. The selected roots were reduced to 10 cm and longitudinally sliced. The necrosis surface of each root piece was scored as a percentage of the total surface of the root piece (Speijer and De Waele, 1997).

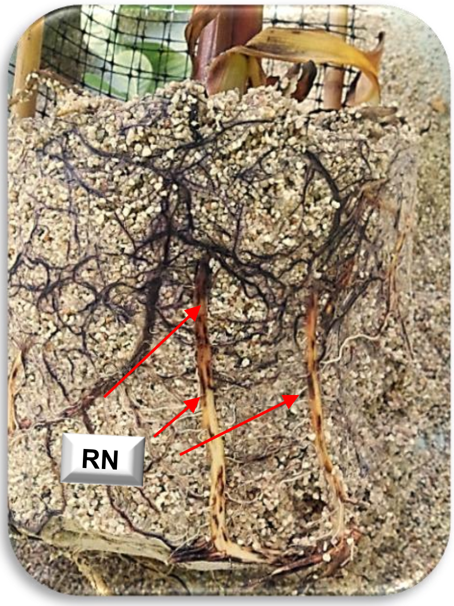


Figure 20: Roots of inoculated banana

RN: Roots necrosis indicating nematodes damages (red arrows)

4.4. Statistical analysis

Different statistical analyses were performed with the SAS Enterprise Guide packages. Some data were normalized prior to statistical analysis. Data of AMF root colonization (i.e. %A, %V and %RC) were arcsin (x/100) transformed and data for nematodes population were log(x + 1) transformed.

ANOVA I, II or III were performed. Normal distributed data were tested with Tukey HSD (Honestly Significant Difference) for multiple comparisons of group means.

Chi square test were performed for nematodes chemotaxis data analysis with Bonferroni correction ($p \leq 0.05$) to compare nematodes distribution between roots exudates directions. Kruskal-Wallis test was performed when analyzing nematodes viability in roots exudates and significant different data ($p \leq 0.05$) were tested with Kruskal-Wallis Chi square.

V. RESEARCH RESULTS

V. RESEARCH RESULTS

CHAPTER 1

Rhizophagus irregularis MUCL 41833 decreases the reproduction ratio of *Radopholus similis* in the banana cultivar Yangambi km5

Abla Anene, Marie Chantal Koffi, Christine Vos and Stéphane Declerck (2013)

Adapted from the research article published Nematology 15: 629-632

Preface

In chapter 1, we investigated the effects of the AMF *Rhizophagus irregularis* MUCL 41833 on the population density of *Radopholus similis* (i.e. number of juveniles, males and females) in roots and damages (root necrosis index) caused by this nematode on the root system of the partially resistant banana cultivar Yangambi km5. This experience was conducted as a continuation of the work of Koffi *et al.* (2013a) who investigated for the first time, under strict *in vitro* culture conditions, the effects of *R. irregularis* MUCL 41833 on the reproduction of *R. similis* in the highly-susceptible banana cultivar (Grande Naine). With our study, it was the objective to determine whether a partially-resistant cultivar (yangambi km5) would benefit more from the AMF than a highly-susceptible cultivar (Grande Naine) or vice-versa.

INTRODUCTION

Bananas are susceptible to various root nematodes among which the burrowing nematode *Radopholus similis* is one of the most damaging. The control of this migratory endoparasite is mostly conducted via the application of nematicides as well as on agricultural practices such as fallow, paring and hot water treatment of the corms, the use of resistant cultivars and the large-scale micropropagation of plantlets (Quénehervé, 2009). The application of bio-control organisms is another option nowadays considered as a potential alternative to decrease the damages caused by nematodes (Siddiqui and Mahmood, 1996, 1999; FAO, 1997).

Arbuscular mycorrhizal fungi (AMF) are key microorganisms of the rhizosphere. They form symbiotic associations with nearly 80% of plant species, among which bananas. They improve plant nutrition and have also been reported to decrease nematode infestation. Elsen *et al.* (2001) demonstrated that *Rhizophagus irregularis* MUCL 41833 was able to decrease the reproduction capacity of *R. similis* in excised root organs of carrot (i.e. in root organ culture – ROC). More recently, Koffi *et al.* (2009) developed an *in vitro* culture system associating autotrophic micropropagated banana plantlets with *R. irregularis* MUCL 41833. With a closely-related *in vitro* cultivation system, Koffi *et al.* (2013a) were the first to investigate the impact of *R. irregularis* MUCL 41833 on the resistance/tolerance of Grande Naine, a banana cultivar particularly sensitive to *R. similis*. They observed a decrease by 60% and 56% of the nematode population and surface of root necrosis, respectively, in the plantlets associated to the AMF. Here, Yangambi km5, a partially resistant cultivar to *R. similis*, was associated to *R. irregularis* MUCL 41833 under strict *in vitro* culture conditions and challenged with *R. similis*. The effects of the AMF on the reproduction ratio of the nematodes as well as the impact of the latter on the AMF were studied.

MATERIAL AND METHODS

Tissue-cultured banana (*Musa acuminata*) plantlets, c.v. Yangambi km5 were provided by the International Musa Germplasm Collection at INIBAP (KUL, Belgium). The plantlets were proliferated, regenerated and rooted as in Koffi *et al.* (2013a). A mycelium donor plant (MDP) *in vitro* culture system was set up (see Koffi *et al.*, 2013a), consisting of a bi-compartmented Petri plates with a root compartment (RC) and a hyphal compartment (HC). The two compartments were filled with 100 ml Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998) without sucrose and vitamins and supplemented with 10 ml.l^{-1} MES buffer (MSR$_{SS}$+MES) and solidified with 3 g.l^{-1} Phytagel (Koffi *et al.*, 2013a). Seeds of *Medicago truncatula* Gaertn. C.v. Jemalong A17 (SARDI, Australia) were surface sterilized (see Koffi *et al.*, 2013a) and plantlets subsequently placed in the RC of each MDP *in vitro* culture system (for details, see Koffi *et al.*, 2013b). Half of the plantlets were inoculated with 100 spores of *R. irregularis* (+AMF treatment), the other half remaining free of AMF (-AMF treatment). The Petri plates were placed in an incubator (21/18°C (day/night), 16h illumination, 70% relative humidity and 300 µmol m^{-2}s^{-1} photosynthetic photon flux). Starting from the third week, the RC received weekly 10 ml of MSR$_{SS}$ + MES medium. After four weeks incubation, the mycelium of the AMF crossed the partition wall separating the RC from the HC and proliferated in the HC. Four banana plantlets (18 days old) were plated in the HC of each MDP *in vitro* culture system (for details, see Koffi *et al.*, 2013b). In parallel, 4 banana plantlets were placed in the control systems (i.e. –AMF treatment). The systems were placed in the growth chamber (27/25 °C day/night, 12h illumination, 70% humidity and 300 µmol m^{-2}s^{-1} photosynthetic photon flux). The *M. truncatula* plantlets in the RC received weekly 10 ml of MSRss+MES medium. After three weeks, the banana plantlets were transferred into individual systems (Koffi *et al.*, 2013b) on the same growth medium as above. The Petri plates were incubated in a growth chamber under the same conditions as above. Every week, the systems received 50 ml of

MSR_{SS}+MES medium solidified with 3 g.l^{-1} Phytagel. After another 3 weeks, 6 plantlets per treatment were randomly harvested to evaluate root colonization by the AMF. Half of the remaining plantlets (i.e. 12) of both treatments were inoculated with 100 aseptically produced juveniles and adults of *R. similis*. The systems were maintained for 6 weeks and 50 ml of MSR_{SS}+MES medium was added every week. Four treatments, each with 6 replicates, were thus considered: the mycorrhizal plants inoculated (+AMF+Nem) or not (+AMF-Nem) with nematodes and the non-mycorrhizal plants inoculated (-AMF+Nem) or not (-AMF-Nem) with nematodes. AMF root colonization was assessed by the method of McGonigle *et al.* (1990) following staining (Vierheiling *et al.*, 1998). To evaluate nematode reproduction, roots of infected banana plantlets were cut in 1 cm pieces, macerated with water in a blender for 30 sec with three bursts of 10 sec intervals. The nematodes were separated from the root tissue and debris using a series of sieves (250-100-50-5µm) (Speijer and De Waele 1997) while nematodes in the culture medium were collected using modified Bearmann funnel (Coyne et al., 2010). To normalize the values, the number of nematodes was log(x+1) transformed while root colonization by the AMF was arcsin(x/100) transformed. The data were subsequently analyzed by ANOVA and the means were separated by the Tukey test ($P<0.05$) with the CoStat package.

RESULTS AND DISCUSSION

At the time of nematode inoculation, the root colonization (estimated on 6 randomly harvested banana plantlets) was 32.6 ± 16% with 7.2 ± 6% arbuscules and 14.6 ± 8% spores/vesicles. At the end of the experiment (6 weeks later), the root colonization had increased significantly whatever the presence/absence of nematodes. No significant differences were noted in the percentage of root colonization and percentage of spores/vesicles between the +AMF+Nem (i.e. 77.3 ± 15% and 23.3 ± 5%, respectively) and +AMF-Nem treatments (i.e. 67.2 ± 14% and 25.8 ± 11%, respectively). To the contrary, the percentage of arbuscules was significantly higher in the +AMF+Nem treatment (i.e. 58.8± 23%) as compared to the +AMF-Nem treatment (i.e. 14.3 ± 3).

The number of nematodes extracted from the roots and MSR$_{SS}$+ MES medium are reported in Table 1. All the developmental stages were observed in the roots and medium. In presence of AMF, the population of *R. similis* was reduced by 52.6% as compared to the control. However, this reduction was not significant for all developmental stages. Within the roots, no differences were noted for the number of eggs, males and females, while a significantly lower number of juveniles was noted in presence of AMF. Interestingly, the total number of nematodes in the roots was 43.5% lower in the +AMF+Nem treatment as compared to the –AMF+Nem treatment. In the medium, the number of eggs (due to decaying roots) as well as the number of males did not differ between the treatments, while the number of females and juveniles were significantly higher in the medium of the –AMF+Nem treatment. The total number of nematodes in the medium was 64.4% lower in the +AMF+Nem treatment as compared to the –AMF+Nem treatment.

Both the AMF and nematodes were able to complete their life cycle in the presence of *in vitro* banana plantlets. However, the number of nematodes summed over the roots and medium was decreased by 52.6% in presence of the AMF, while root colonization (i.e. % total hyphae and % spores/vesicles) appeared not influenced by the nematode. Curiously, the % arbuscules was significantly increased in presence of the nematodes.

The lower multiplication rate of *R. similis* in the roots of Yangambi km5 observed in our experiment corroborated earlier studies of Speijer and De Waele (1997) and Fogain and Gowen (1998). These authors reported on the difficulty of *R. similis* to multiply on Yangambi km5 in greenhouse condition; but according to Elsen and De Waele (2004) the resistance/tolerance of

Yangambi km5 is not that clear under *in vitro* culture conditions. A reduced number of juveniles was

Table 1: Nematode population densities in the roots of mycorrhizal (+AMF) and non-mycorrhizal (–AMF) *Musa acuminata* cv. Yangambi km5 plantlets grown under in vitro culture conditions, six weeks after inoculation with 100 aseptically produced juveniles and adults of *R. similis* (Nem)

	No. of nematodes in roots					**No. of nematodes in medium**					**Pf**	**Rr**
	Eggs	J	F	M	Total	Eggs	J	F	M	Total		
+AMF+Nem	53±40a	30±18b	31±21a	16±12a	130±20b	8±9a	30±14b	22±14b	3±5a	63±30b	193±37b	1.93b
-AMF+Nem	50±37a	101±64a	58±34a	21±16a	230±83a	3±5a	118±49a	46±32a	10±6a	177±59a	407±124a	4.07a

Data are represented as mean value ± standard error of 6 plantlets per treatment. All nematode population densities were log(x+1) transformed prior to analysis. J = juveniles, F = Females, M = males, Pf = final population of nematodes in roots and medium, Rr = reproduction ratio: total nematodes recovered from roots and medium relative to the 100 nematodes inoculated. Mean value (± standard error) followed by the same letter, within a column, did not differ significantly ($P \leq 0.05$; Tukey).

noticed in the roots and in the medium of the mycorrhized banana plantlets in our experiment. Identically, the number of females was significantly reduced in the medium. These observations suggested that the plant grown in association with the AMF was less prone to root infestation and/or nematode multiplication.

Arbuscules are intraradical structures involved in the bi-directional exchange of nutrients from the AMF to the plants and carbohydrates from the plant to the fungus. Arbuscules have also been associated with an increase in Jasmonate (JA – Pozo and Azcon-Aguilar (2007)). These lipid-based hormone signals are essential in plant defense against herbivory and plant responses to abiotic and biotic stresses (Farmer and Ryan, 1990). They have also been reported as endogenous signals in mycorrhiza-induced resistance/tolerance (MIR). Pozo and Azcon-Aguilar (2007) observed that cells of tomato roots containing arbuscules had an increased level of endogenous JA. These plants presented some resistance/tolerance to *Phytophthora parasitica* infection. Cordier *et al.* (1998) further observed the inability of *P. parasitica* hyphae to invade arbuscules-containing host cells of tomato. The significant increase in % arbuscules observed in the banana plantlets confronted to the nematodes could therefore be a defense response modulated by the plant to increase its resistance/tolerance to the nematode.

As a conclusion, our results demonstrated that the partially-resistant cultivar (Yangambi km5) may benefit from the early colonization (i.e. prior to any contact with the nematode) by an AMF (i.e. *R. irregularis* MUCL 41833) to suppress reproduction of the burrowing nematode *R. similis*.

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CHAPTER 2

The combination of *Crotalaria spectabilis* with *Rhizophagus irregularis* MUCL41833 decreases the impact of *Radopholus similis* in banana.

Abla Anene and Stéphane Declerck (2016)

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Preface

In chapter 1, we investigated the impact of the AMF *R. irregularis* MUCL 41833 on the population of *R. similis* and its damage on banana roots. We observed that almost all developmental stages of the nematode (i.e. number of eggs, juveniles and females) were significantly affected in presence of the AMF. The multiplication rate was drastically decreased, while no effect of the nematode was noticed on the AMF in roots or medium.

Within chapter 2, it was the objective to investigate the combination of this fungus with another strategy often suggested to control nematodes, i.e. the use of push pull plants. Here the effects of *R. irregularis* MUCL 41833 combined with the push pull plant *C. spectabilis* was investigated on the population density and damage of *R. similis* on banana plants. A bi-compartmented microcosm was developed to grow 2 plants in the same pot without having their roots systems mixed. Banana plantlets were pre-mycorrhized in the MDP *in vitro* culture system then transferred in the bi-compartmented microcosm while the seedling of *C. spectabilis* were grown in a pot before their transfer to the system. The nematodes were subsequently inoculated in the compartment containing the banana plants. Root colonization by the AMF and infection by the nematode were simultaneously analyzed in presence/absence of *C. spectabilis*.

Abstract

The past decade has seen substantial progress in our knowledge on microorganisms (e.g. arbuscular mycorrhizal fungi-AMF) and push pull plants (e.g. *C. spectabilis*) that contribute to the biocontrol of nematodes. However, the application of microorganisms together with push pull plants has seldom been considered. Therefore, the objective of the study was to investigate the combined effect of AMF and *C. spectabilis* on the control of the nematode *R. similis* in banana.

Banana plants, pre-colonized or not with the AMF *R. irregularis* MUCL 41833 were grown in 3 L pots in presence/absence of *C. spectabilis*. Above-ground parts were separated with a fixed talpa net to separate shoots and leaves of the two plants. Similarly, the pots were divided below-ground in two compartments by growing the banana roots in a pocket nylon mesh (30 mm), to avoid roots of both plants to intermingle. The banana plants were established first and were followed three weeks later by *C. spectabilis*. Inoculation of nematodes was done in parallel to the planting of *C. spectabilis*. Half of the banana plants received 1000 monoxenically produced juveniles and adults of *R. similis*. Eight treatments were set up with 6 replications: mycorrhizal banana plantlets with/without *C. spectabilis* and with/without nematodes (+ M + C + N, + M + C - N, + M - C + N, + M - C - N) and non-mycorrhizal banana plantlets with/without *C. spectabilis* and with/without nematodes (- M + C + N, - M + C - N, - M - C + N, - M - C - N). Plant growth parameters, root colonization by the AMF and infection by the nematode were evaluated.

R. similis did not impact banana root colonization by the AMF. Conversely, the fungal symbiont as well as *C. spectabilis* significantly decreased the total number of nematodes as well as their multiplication rate. The multiplication rate in the controls (i.e. - M - C + N) was 280.3, while it decreased to 176.5, 106.7 and 83.8 in the - M + C + N, +M - C + N and +M + C + N treatments, respectively. The root necrosis index (RNI) was significantly decreased in presence of the AMF, *C. spectabilis* and the combination of both. The RNI of the control (i.e. - M - C + N) was 61.7% while it was 33.7, 19.8 and 17.2 in the - M + C + N, +M - C + N and +M + C + N treatments, respectively. Banana root fresh weight was significantly increased in presence of *C. spectabilis* and shoot dry weight in presence of AMF, but no increase was noticed in presence of both organisms together. This study reaffirmed that AMF and *C. spectabilis* are effective in decreasing the pressure caused by *R. smilis* in banana roots. It demonstrated further, for the first time, that their combination decreased even more drastically the surface of necrotic cortical tissues caused by the nematodes, opening new avenues for their concomitant use in an integrated pest management strategy.

Key words

Rhizophagus irregularis, Crotalaria spectabilis, Radopholus similis, push pull plant, banana

INTRODUCTION

Bananas and plantain are a major staple food for millions of people (Lassoudière, 2011; FAO, 2014) and an important source of revenues for many countries in the humid and sub-humid tropical regions of the world (Shobhana *et al.*, 2014; CIRAD, 2014). Their production is under threat of several shoot, bunch and root pests (e.g. nematodes and weevil) and diseases (e.g. black Sigatoka, Xanthomonas wilt, Panama Disease) (Ploetz, 2003; AUGURA, 2009; Ngando *et al.*, 2015), although figures on the loose are extremely hard to validate. It is admitted that farmers in the tropics lose much more of their crops due to pests than the EU and USA (estimated 50% and 25 to 30%, respectively) (Maxmen, 2013). The reasons is that pests are a year-round problem in the tropical regions, and farmers are generally poorer and have limited access to adequate control measures (e.g. pesticides, improved varieties).

The burrowing nematode *R. similis* is the most devastating pest in banana around the world (Quénehervé, 2009; Hölscher *et al.*, 2014). It destroys roots, leaving plants with weakened soil anchorage, causing in the most severe case toppling, or reduced capacity to take up and translocate water and nutrients (Gowen *et al.*, 2005). The control of *R. similis* is usually based on nematicides. Even if their utilization has been continuously reduced, their side-effects on the environment and farmers health have encouraged the development of alternative control measures such as fallow, paring and hot water treatment of the corms, the use of resistant cultivars and the planting of *in vitro* micropropagated plantlets (Quénehervé, 2009; CIRAD, 2014).

The application of biocontrol microorganisms (e.g. *Purpureocillium lilacinum*, *Trichoderma viride*, AMF) is another option that proved promising under greenhouse or *in vitro* conditions to control nematodes (Masadeh *et al.*, 2004; Vos *et al.*, 2012; Koffi *et al.*, 2013a; Lopez and Sword, 2015; Panebianco *et al.* 2015). A number of studies have, for instance, reported the impact of AMF in decreasing the populations of *R. similis* in pre-mycorrhized banana (Anene *et al.*, 2013; Koffi *et al.*, 2013a) and tomato (Vos *et al.*, 2012a) plants as well as in excised root organs of carrot (Elsen *et al.*, 2003).

In the last decade, several studies have mentioned the beneficial effects of push pull plants (e.g. *Desmodium uncinatum*, *Tagetes erecta*, *C. spectabilis*.) to control pests (Hassanali *et al.*, 2008; ICIPE, 2011). Push pull plants refers to plants that produce repellents (known as "push" plants) or attractant (known as "pull" plants) semiochemicals (Hassanali *et al.*, 2008; ICIPE, 2011; Pickett *et al.*, 2014;). *Crotalaria spp.* is a legume used as cover crop to control weeds (Wang and McSorley, 2012). Many species of *Crotalaria* are also grown to provide green manure to the soil and are thus used to increase nitrogen content via their symbiosis with rhizobium (Wang *et al.*, 2002). Interestingly, these plants also produce secondary metabolites (i.e. allelochemicals) that are toxic or inhibitory to some pests and provide niches for antagonistic flora and fauna trapping nematodes (Wang *et al.*, 2002). The alkaloid monocrotaline-pyrrolizidine is the main toxic principle of *C. spectabilis* with nematicidal, ovicidal and repellent effects on plant parasitic nematodes (Thoden *et al.*, 2009; Marahatta *et al.*, 2012). *Crotalaria* species are non-host for root-knot nematodes (e.g. *M. incognita*) and poor-hosts for some migratory nematodes (e.g. *P. penetrans*) (Wang *et al.*, 2002; Thoden *et al.*, 2009). The few nematodes that penetrate their roots do not develop beyond the penetration point, limiting their proliferation (Wang *et al*, 2002; Germani and Plenchette 2004). Interestingly, ground seeds of *Crotalaria* species incorporated into the soil at 2% of soil volume suppressed the whole population of the root-knot nematodes *M. javanica* and *M. incognita* (Rich and Rahi, 1995).

Germani and Plenchette (2004) reported that *Crotalaria* species are highly mycotrophic plant and suggested that these plants could be used as pre-crops for providing green manure while at the same time decreasing the level of detrimental nematodes and increasing the level of beneficial AMF in soil. The combination of *Crotalaria* species and AMF could thus represent an interesting option to control/decrease the impact of nematodes in banana. To the best of our knowledge, no study reported on such combination for the biocontrol of *R. similis* in banana plants.

The objective of the present study was to investigate the combination of an AMF, *R. irregularis* MUCL 41833 with *C. spectabilis*, on the population of *R. similis* in banana (*M. accuminata* cv. Grande Naine, AAA genome, Cavendish group) plantlets grown under greenhouse conditions. Root colonization by the AMF and infection by the nematode were simultaneously analyzed in presence/absence of *C. spectabilis* to evaluate the impact of the AMF, *C. spectabilis* and both in combination on the nematodes and the impact of the later on the fungal symbiont and push pull plant.

MATERIALS AND METHODS

Biological material

Tissue-cultured banana plantlets (*M. acuminata* Colla c.v. Grande Naine, clone CV902, AAA genome, Cavendish group) were provided by VITROPIC SA (Montpellier, France). The plant material was proliferated, regenerated and rooted on the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 30 g.l^{-1} sucrose and 2 g.l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA), and with pH adjusted between 6.12 and 6.15 before sterilization (Banerjee and De Langhe, 1985). The plantlets were incubated in a growth chamber at 27/25 °C (day/night) with a photoperiod of 16 hday^{-1} and under a photosynthetic photon flux of 300 µmol m^{-2}s^{-1}.

Seeds of *C. spectabilis* Roth (Wolfseeds, Brazil) were sown in 1 l pots containing 500 g of sterilized sand (2 x 8 hours at 110°C) (1-2 mm diameter; Euroquartz, Belgium) and maintained in a growth chamber at 28/24°C (day/night) with a relative humidity of 80% during the first 2 weeks and 70% thereafter. The pots were watered with deionized water when needed. The seedlings were kept in the pots for 3 weeks reaching a size of 10 cm at the start of the experiment.

Seeds of *M. truncatula* Gaertn. c.v. Jemalong strain A17 (SARDI, Australia) were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min, rinsed in sterilized (121 °C for 15 min) deionized water and further germinated in 90 mm Petri plates filled with 35 ml of the Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998) solidified with 3 g.l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA) following the method of Declerck *et al.* (1998). The Petri plates were incubated in the dark at 27°C for 4 days.

A strain of *R. irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 (former *Glomus intraradices*) was supplied by GINCO (http://www.mycorrhiza.be/ginco-bel/index.php) on Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 grown in 90 mm Petri plates on the MSR medium (Declerck *et al.*, 1998). The Petri plates were maintained in the dark in an inverted position at 27 °C until thousands of spores were obtained.

A strain of *Bradirhizobium arachidis* Wang, Chang, Zheng, Zhang, Zhang, Sui, Wang, Hu, Zhang and Chen 2013 LMG 26795 was supplied by BCCM/LMG (http://bccm.belspo.be/about/lmg.php). The bacteria was cultured in 90 mm Petri plates on Yeast Malt Agar (YMA). A subsample was then isolated from a one week-old culture and poured in a flask containing 100 mL liquid Difco^{TM} Yeast Malt (YM) Broth medium. The flask was maintained in agitation at 80 rpm during 10 days, at 28°C in the dark for inoculum production.

A strain of *R. similis* (Cobb) Thorne originating from Uganda (high virulent strain) was provided by the Nematology laboratory of K.U. Leuven (Belgium). The nematodes (juveniles and adults) were maintained under aseptic conditions on carrot discs (Pinochet *et al.*; 1995) at 28°C in the dark.

Experimental set up

Pre-mycorhization of banana plantlets

Three weeks-old *in vitro* produced banana plantlets (5cm in size with 3 leaves and 3 roots of 7-10 cm length) were placed in the Mycelium Donor Plant (MDP) *in vitro* culture system (Voets *et al.*, 2005) as described by Anene *et al.* (2013) for mycorrhization. Briefly, bi-compartmented systems were set up with a root compartment (RC) containing the AMF associated to *M. truncatula* and a hyphal compartment (HC) in which only the extraradical mycelium (ERM) of the AMF was allowed to grow. The RC and HC contained respectively 15 ml and 100 ml MSR medium without sucrose and vitamins and supplemented with 10 ml 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (MSR_{SS}+MES) and solidified with 3 $g.l^{-1}$ Phytagel. Following development of the ERM in the HC (within 6 weeks), three banana plantlets were introduced per MDP *in vitro* culture system with their roots plated on the MSRss+MES medium in contact with the ERM. The plantlets were incubated for 3 weeks. The same number of plantlets was placed in similar systems without AMF, as controls. The systems were then incubated in a growth chamber (27/25 °C day/night, 12h illumination, 70% relative humidity and 300 µmol $m^{-2}s^{-1}$ photosynthetic photon flux). MSRss+MES medium (20 ml) was added weekly in the HC of each system.

Establishment of compartmented microcosms and planting of the banana plantlets

The experiment design was a completely randomized block with 6 replications of each treatment. Eight treatments were thus set up: Mycorrhizal banana plantlets with/without *C.*

spectabilis and with/without nematodes (+M+C+N, +M+C-N, +M-C+N, +M-C-N) and non-mycorrhizal banana plantlets with/without *C. spectabilis* and with/without nematodes (-M+C+N, -M+C-N, -M-C+N, -M-C-N).

Microcosms consisting of pairs of plants (banana and *C. spectabilis*) were set up in 3 l pots divided above-ground by a fixed talpa net (17x17 mm, Howitec Talpa Net, Nederland) to separate shoots and leaves of the two plants. The pots were further divided below-ground in two compartments to avoid roots of both plants to intermingle. In the first compartment, representing 1/3 of the pot volume, a pocket of nylon mesh (30µ) was buried in the pot and filled with 0.8 kg sand (1-2 mm diameter; Euroquartz, Belgium), while the second compartment was filled with 1.6 kg sand. The 1st and 2nd compartments were used to grow *C. spectabilis* and banana, respectively. Thirty pre-colonized banana plantlets and the same number of non-mycorrhizal banana plantlets were planted in the 2nd compartment of each pot. The plantlets received weekly 50 ml of Long Ashton (Hewitt, 1966).

The banana plantlets were maintained in a growth chamber at 28/ 24°C (day/night) with a relative humidity of 80% during the first 2 weeks and 70% thereafter. The pots were watered every 2 days with approximately 150 ml of deionized water.

Inoculation of nematodes and planting of *C. spectabilis*

After 3 weeks of acclimatization, 6 mycorrhizal and non-mycorrhizal banana plantlets were harvested to evaluate root colonization. The remaining pots (24 with and 24 without pre-mycorrhized bananas) were divided in four homogenous groups based on banana plant height and leaf area. Two groups (12 pots with pre-mycorrhized bananas and 12 non-mycorrhizal bananas) were inoculated with 1000 monoxenically- produced juveniles and adults of *R. similis*, the other two groups remained free of nematodes. The nematodes were collected from the carrot discs and diluted in sterilized water to prepare an aliquot of 200 nematodes per ml. Two holes of 2 cm depth were made near the bulb of the banana plantlet and 5 ml (i.e. 1000 nematodes) of the aliquot was introduced with a syringe. At the same time, three week old seedlings of *C. spectabilis* (± 7.5 cm height) were transplanted in half of the pots (i.e. 6 pots) from each group (Figure 2). After 3 days, the seedlings were inoculated with 5 mL suspensions of *B. arachidis* (10^9 bacterial cells). Pots without *C. spectabilis* received 5ml of culture medium (Difco^{TM} Yeast Malt (YM) Broth.

Plant growth parameters

At the end of the experiment (i.e. after 11 weeks of growth in the pots), root fresh weight (RFW) and shoot dry weight (SDW) were evaluated on banana and *C. spectabilis. S*hoots were dried in oven at 50°C during one week. Roots of banana and *C. spectabilis* were separated in two equivalent parts to evaluate root colonization by the AMF and root infection by the nematodes.

Root colonization

Root colonization was evaluated at three times: (1) 3 weeks following plating of the banana plantlets into the extraradical mycelium network *in vitro*, (2) 3 weeks after transfer into the pots (i.e. at the time of nematode inoculation) and (3) at harvest (i.e. 11 weeks after transfer into the pots). The roots were stained with ink (Parker®) and vinegar (Vierheilig *et al.* (1998). Roots were first cleared 45 minutes in KOH (10%) and 30 minutes in H_2O_2 (3.5% for banana roots) at 70°C and rinsed several times with tap water. Then the cleared roots were stained 30 minutes with 5% ink-vinegar solution with white household vinegar (5% acetic acid). Root colonization was assessed by the method of Mc Gonigle *et al.* (1990). 150 intersections were observed under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 20-40×

magnifications. Total root colonization (% RC), abundance of arbuscular colonization (% A) and intraradical spores/vesicles (% V) were determined.

Nematode population

Root necrosis index (RNI) was determined following the method of Speijer and De Waele (1997) by scoring 10 x 10 cm longitudinally sliced functional primary roots randomly selected. Nematode reproduction was also evaluated. Twenty five g fresh roots of inoculated banana and *C. spectabilis* were cut in 1 cm pieces and macerated in water with a blender for 30 sec. with three bursts of 10 sec. intervals. The nematodes were separated from the root tissues and debris using a series of sieves (250-100-25 µm). The nematodes were collected from the 25 µm sieve with distilled water. The number of juveniles, females, males and total number of nematodes per plant and the reproduction ratio (final population/initial population) were determined.

Nodule evaluation

The number of nodules on *C. spectabilis* roots was evaluated. Five g of randomly selected roots of *C.spectabilis* were weighted and the number of nodules enumerated. The total number of nodules per plant was then extrapolated to the roots weight of the plant.

Statistical analysis

To normalize the values, the number of nematodes was log(x+1) transformed prior to statistical analysis. For root colonization by the AMF, %A, %V and %RC were arcsin(x/100) transformed prior to statistical analysis.

Plant data as well as root colonization parameters by AMF and infestation by nematodes were analyzed by ANOVA I, II and III and means separated by the Tukey Test ($P<0.05$) with the SAS Enterprise Guide package.

RESULTS

Plant growth parameters

The RFW and SDW of banana plants are presented in Table 1. No significant effect of AMF was noticed on RFW (Table 2). To the contrary a significant effect of nematodes and *C. spectabilis* was observed on this parameter. The banana plants in the treatments with nematodes had lower RFW, while the reverse was noticed for the banana plants in the treatments with *C. spectabilis*. SDW of the banana plants was significantly increased in presence of AMF, while no effect was noticed in presence of nematodes or *C. spectabilis*. For both RFW and SDW, no significant interaction was noticed between AMF and *C. crotalaria* or nematodes, between *C. crotalaria* and nematodes or between AMF, *C. spectabilis* and nematodes.

The RFW and SDW of *C. spectabilis* are presented in Table 3. A significant effect of AMF and nematodes was observed on RFW. The *C. spectabilis* plants in the treatments with AMF as well as with nematodes had higher RFW. SDW was significantly decreased in presence of nematodes, while no effect was observed for AMF. For both RFW and SDW, not significant interaction was observed between AMF and nematodes.

Table 2 : Observed data of Root fresh weight (RFW), and shoot dry weight (SDW) of mycorrhizal banana plantlets grown in presence/absence of *C. spectabilis* and infested or not with *R. similis* (+M+C+N, +M+C-N, +M-C+N, +M-C-N) and non-mycorrhizal banana plantlets grown in presence/absence of *C. spectabilis* and infested or not with *R. similis* (-M+C+N, -M+C-N, -M-C+N, -M-C-N)..

Treatment	RFW (g)	SDW (g)
–M – C + N	77.8 ± 12	18.1 ± 2
+M – C + N	93.4 ± 18	20.7 ± 3
+M + C + N	80.7 ± 6	20.6 ± 2
–M + C + N	92.6 ± 21	18.3 ± 3
–M + C – N	102.1 ± 20	19.2 ± 3
+M – C – N	105.9 ± 11	19.4 ± 1
–M – C – N	112.1 ± 11	19.2 ± 2
+M + C – N	124.7 ± 17	21.9 ± 3
Effects (p-values)		
AMF	0.2582	0.0118**
C. spectabilis	0.0445*	0.3516
Nematodes	<.0001***	0.5323
AMF X *C. spectabilis*	0.1339	0.434
AMF X nematodes	0.4739	0.4772
***C. spectabilis* X nematodes**	0.2902	0.4506
AMF X *C. spectabilis* X nematodes	0.0855	0.4026

Data are mean values ± standard errors of 6 plants. Main effects and interaction between the factors: “AMF”, “*C. spectabilis*”, “Nematodes” are presented (P ≤ 0.05, Tukey test). Tests that revealed significant differences at values below 0.05, 0.01 and 0.001 α level are followed by *, **, ***, respectively.

Table 3: Observed data of Root fresh weight (RFW) and shoot dry weight (SDW) and number of nodules on roots of *C. spectabilis* plants grown in presence/absence of AMF and *R. similis*. (+M+C+N, +M+C-N, -M+C+N, -M+C-N).

Treatment	RFW (g)	SDW (g)	Nb nodules
+ M + C + N	33.1 ± 9	10.2 ± 2	1265 ± 1700
+ M + C– N	27.1 ± 6	12.1 ± 1	908 ± 401
- M + C + N	23.7 ± 4	11.1 ± 1	381 ± 95
- M + C– N	18 ± 2	12.8 ± 2	683 ± 434
Effects (p-values)			
AMF	0.0011**	0.2364	0.148
Nematodes	0.0243*	0.0088**	0.941
AMF X nematodes	0.9674	0.8977	0.381

Data are mean values ± standard errors of 6 plants. Main effects and interaction between the factors: “AMF” and “Nematodes” are presented ($P \leq 0.05$, Tukey test). Tests that revealed significant differences at values below 0.05, 0.01 and 0.001 α level are followed by *, **, ***, respectively.

AMF root colonization of banana and *C. spectabilis*

Three weeks after the introduction of the *in vitro* produced banana plants into the ERM network of the MDP *in vitro* culture system, 65 ± 13 % of the roots were colonized with an average of 21.2 ± 13 % of spores/vesicles and 12.8 ± 9 % of arbuscular colonization of the roots. Three weeks later (i.e. at the time of nematode inoculation), total root colonization was 79 ± 14 % with an average of 42 ± 9 % of spores/vesicles and 34.3 ± 16 % of arbuscular colonization.

At banana harvest, no effects of nematodes or *C. spectabilis* was observed on %RC and %A, while both factors in combination significantly impacted %A (Table 4). The Post-hoc analysis ($P \leq 0.05$; Tukey Test) revealed that %A was the highest in the treatment with *C. spectabilis* and nematodes, while no difference was noticed between the three other treatments. This combination did not influence %V neither did nematodes. To the contrary, in presence of *C. spectabilis* the %V was significantly increased (Table 4).

Table 4: Observed data of percentage of arbuscules, spores/vesicles and total colonization by AMF in mycorrhizal banana plants grown in presence/absence of *C. spectabilis* and infested or not with *R. similis* (+M-C+N, +M+C+N, +M+C-N, +M-C-N)

Treatment	**% arbuscules**	**% spores/vesicles**	**% colonisation**
+M – C + N	35 ± 23	39 ± 13	74.81 ± 9
+M + C + N	10 ± 5	65.16 ± 16	78.33 ± 15
+M + C – N	13.67 ± 11	63.33 ± 9	73.5 ± 7
+M – C – N	9.8 ± 3	34.33 ± 16	79.33 ± 11
Effects (p-values)			
C. spectabilis	0.0573	0.0001***	0.8006
Nematodes	0.0694	0.5044	0.9712
***C. spectabilis* X nematodes**	0.0128**	0.8196	0.3181

Data are mean values ± standard errors of 6 plants. Main effects and interaction between the factors: "*C. spectabilis*" and "Nematodes" are presented ($P \leq 0.05$, Tukey test). Tests that revealed significant differences at values below 0.05, 0.01 and 0.001 α level are followed by *, **, ***, respectively.

Root colonization of *C. spectabilis* was noticed in presence as well as in absence of nematodes (Table 5). However, no significant differences were observed in the %A, %V and %RC between the treatments (Table 5).

Table 5: Observed data of percentage of arbuscules, spores/vesicles and total colonization by AMF in roots of *C. spectabilis* in pots of mycorrhizal banana plants grown in presence/absence of *R. similis* (+M+C+N, +M+C-N).

Treatment	% arbuscules	% spores/vesicles	% colonization
+ M + C + N	15.33 ± 11 **a**	36.67 ± 12 **a**	61.5 ± 23 **a**
+ M + C– N	19 ± 11 **a**	25.5 ± 15 **a**	43.67 ± 20 **a**

Data are mean values ± standard errors of 6 plants. Data followed by the same letter, within a column, did not differ significantly (P ≤ 0.05; Tukey Test).

Nematode infestation of banana and *C. spectabilis* roots

The number of nematodes (juveniles, females, and males), their multiplication rate and RNI are presented in Table 6. All the development stages of *R. similis* were significantly impacted by AMF. The number of juveniles, females and males as well as the total number of nematodes was significantly reduced in the colonized banana roots. This resulted in a significantly lower multiplication rate as well as RNI in the treatments with AMF as compared to the treatments without AMF. Juveniles and total number of nematodes in banana roots, were significantly impacted by *C. spectabilis*. In presence of *C. spectabilis*, the number of juveniles and the total number of nematodes in banana roots was significantly decreased as compared to the treatments without *C. spectabilis*, while no significant impact of *C. spectabilis*, was noticed on the number of females and males. Similarly to AMF, *C. spectabilis* significantly decreased the nematodes multiplication rate and the RNI. The number of females as well as the RNI was significantly decreased in presence of AMF and *C. spectabilis* together. Post-hoc analysis ($P \leq 0.05$; Tukey Test) revealed that AMF X *C. spectabilis* had greater impact in decreasing females' population as well as in reducing RNI. To the contrary, no significant impact of both factors in combination was observed on the number of juveniles, males and total number of nematodes in the banana roots.

Whatever the presence or absence of AMF, no nematode symptoms were observed on the roots of *C. spectabilis.* No juveniles were observed in the root of *C. spectabilis* in presence of AMF*,* while in absence of AMF, 117 ± 160 juveniles were enumerated. The number of female (133 ± 41 and 358 ± 447 in presence or absence of AMF, respectively), male (25 ± 27 and 42 ± 80 in presence or absence of AMF, respectively) and total number of nematodes (158 ± 58 and 517 ± 661 in presence or absence of AMF, respectively) did not differ significantly in presence of AMF.

Number of nodules in *C. spectabilis*

The number of nodules on the roots of *C. spectabilis* was more important in presence of AMF than in absence (Table 3). However, this numbers did not differ significantly between treatments.

Table 6: Nematode population densities, multiplication rate and root necrosis index (RNI) in the roots of mycorrhizal banana plants grown in presence/absence of *C. spectabilis* (+M+C+N, +M-C+N) and non-mycorrhizal banana plantlets grown in presence/ absence of *C. spectabilis* (-M+C+N, -M-C+N). Data are reported for 25g of banana roots.

Treatment	**Juveniles**	**Females**	**Males**	**Total**	**Multiplication rate**	**RNI (%)**
+M + C + N	10067 ± 2767	15079 ± 5385	596 ± 252	25741 ± 7864	83.8 ± 28	17.2 ± 5
+M – C + N	14742 ± 2736	13917 ± 4717	600 ± 252	29258 ± 4972	106.7 ± 10	19.8 ± 7
–M + C + N	22625 ± 4887	23325 ± 8350	1325 ± 761	47275 ± 13172	176.5 ± 70	33.7 ± 5
–M – C + N	44425 ± 17106	47650 ± 18917	1425 ± 1149	93500 ± 35078	280.3 ± 82	61.7 ± 8
Effects (p-values)						
AMF	<.0001***	<.0001***	0.0142**	<.0001***	<.0001***	<.0001***
C. spectabilis	0.0003***	0.0561	0.9992	0.0039**	0.0121**	<.0001***
AMF x *C. spectabilis*	0.3459	0.0225*	0.9807	0.0589	0.0934	0.0002***

Data are mean values ± standard errors of 6 plants. Multiplication rate= total nematodes recovered from roots relative to the 1000 nematodes inoculated. Main effects and interaction between the factors: “AMF” and “*C. spectabilis*” are presented (P ≤ 0.05, Tukey test). Tests that revealed significant differences at values below 0.05, 0.01 and 0.001 α level are followed by *, **, ***, respectively.

DISCUSSION

Arbuscular mycorrhizal fungi as well as *C. spectabilis* have been reported to partially control nematodes in numerous studies (Wang *et al*, 2002; Germani and Plenchette 2004; Vos *et al.*, 2012a; Koffi *et al.*, 2013a; Anene *et al.*, 2013). However, their concomitant application has never been considered. In the present study, banana plants associated or not to AMF and grown in presence or not of *C. spectabilis* were challenged by the burrowing nematode *R. similis* under pot culture conditions. Root fresh weight and shoot dry weight as well as colonization by the AMF and infection by the nematodes were evaluated. RFW and SDW of bananas were increased in presence of *C. spectabilis* and AMF, respectively, both in absence or presence of the nematodes. Banana root colonization by the AMF was high and not impacted by the nematode. Similarly, root colonization of *C. spectabilis* was important and not influenced by *R. similis*. To the contrary, the multiplication rate of nematodes in banana roots was affected by AMF or *C. spectabilis*. The surface of necrotic cortical tissues of bananas was strongly decreased in presence of AMF and *C. spectabilis* used alone and was even more reduced in presence of both in combination.

The banana plantlets were heavily colonized at transfer from *in vitro* to the pot systems, as earlier reported by Oye Anda *et al.* (2015). This supported that the development of numerous hyphae extending from a mycorrhized plant (e.g. *M. truncatula*) is an adequate source of inoculum for the fast and heavy colonization of banana roots under *in vitro* culture conditions (Koffi *et al.*, 2013a). Root colonization remained high in the pots and was not influenced by the presence of *R. similis*. This was previously mentioned by Elsen *et al.* (2001), Koffi *et al.* (2013a) and Anene *et al.* (2013) under *in vitro* culture conditions and repeated in the present experiment in pot conditions. This suggested that pre-colonization of banana plants prior to any contact with *R. similis* is a serious option to consider in the control of this burrowing nematode as suggested by Cheng *et al.* (2001).

In presence of *C. spectabilis*, the abundance of vesicles/spores in banana roots increased drastically, while no effect was noticed on the number of arbuscules or total colonization. The reason for this observation is unknown but it is not excluded that root exudates of *C. spectabilis* had a stimulatory effect on the production of these reserve and reproductive structures by a direct effect on the AMF or an indirect effect via the banana plant.

Root colonization by AMF was also observed in *C. spectabilis* demonstrating the development of a common mycelium network linking the pre-mycorrhized banana to *C. spectabilis*. The AMF was able to spread from the banana roots in the pot volume, crossing the nylon mesh and extending in the compartment occupied by *C. spectabilis*. Root colonization was high, within a period of 5 weeks, with vesicles/intraradical spores and arbuscules formed. This confirmed the high mycotrophic status of *C. spectabilis*, as earlier reported by Germani and Plenchette (2004) and the role of extraradical mycelium extending from a mycorrhized plants to colonize rapidly a neighbor plant *in vitro* (Voets *et al.*, 2005) and in pots (Merrild *et al.*, 2013).

Similarly to the banana plants, the presence of nematodes did not impact root colonization of *C. spectabilis*. This suggested that the concomitant inoculation of AMF and nematodes was not detrimental to root colonization by the fungus, even though it is important to notice that *C. spectabilis* is non host or poor host to nematodes (Wang *et al.*, 2002; Thoden *et al.*, 2009). To the contrary, the population of nematodes in the roots of *C. spectabilis* decreased by 70 % in presence of AMF and no juveniles were enumerated. The absence of juveniles may be related to the synthesis and exudation of monocrotaline, a secondary metabolite of *C. spectabilis*, which was reported to inhibit the hatching of eggs in *M. incognita* and *P. penetrans* (Thoden *et al.*, 2009). It is not excluded that the effect of this secondary metabolite was amplified by the concomitant presence of AMF in the roots as suggested by the total absence of juveniles in AMF-colonized plantlets.

The number of nodules on the roots of *C. spectabilis* was increased in presence of AMF, although non-significantly. This was similarly reported by Germani and Plenchette (2004) with different species of *Crotalaria* and described by Singha and Sharma (2013) in *C. pallida* and Bath *et al.* (2010) in *Vigna radiate.* These authors suggested a stimulatory effect of AMF on the production of nodules. In our study, *C. spectabilis* was colonized by AMF via a common mycelium network extending from the banana plants and the period of contact may have been too short to significantly stimulate the production of nodules in *C. spectabilis*, although their number was increased. Likewise, no impact of nematodes was noticed on the number of nodules. This was earlier reported by Wang *et al.* (2002) and Germani and Plenchette (2004) and attributed to the poor-host behavior of *C. spectabilis*.

Banana SDW was increased when associated to AMF, in absence as well as in presence of the nematodes, while no effect was noticed on root dry weights. The increase in SDW has been reported in numerous studies and often attributed to an improved nutrient content (especially P) in shoots (Declerck *et al.*, 1995; Declerck *et al.*, 2002a, Fogain *et al.*, 2001). This was attributed to the capacity of AMF to increase the nutrient absorbing surface of the roots by hundredfold, thus enabling the plants to efficiently exploit nutrient resources not available otherwise (Larcher, 2003). Interestingly, Koffi and Declerck (2015) demonstrated that the *in vitro* pre-mycorrhization of banana plantlets significantly increased their SDW during acclimatization in the greenhouse. This procedure was similar in the present experiment and confirmed the interest of the early/pre-mycorrhization of plantlets before transfer to greenhouse or field and possible interaction with below-ground pests such as nematodes. The increase in SDW was not accompanied by an increase in RFW. RFW of the banana plants was significantly impacted by *R. similis*. This is most often related to the feeding of nematodes on the roots, impacting their growth and biomass production (Elsen *et al.*, 2008; Quénehervé, 2009). Interestingly, this impact was compensated by the presence of AMF as suggested by the increase in SDW of the pre-mycorrhized banana plants (Pozo and Azcon-Aguilar, 2007; Elsen *et al.*, 2008).

RFW of *C. spectabilis* was increased in presence of AMF, while no significant impact was noticed on SDW. RFW of *C. spectabilis* was increased in presence of nematodes, even if the number of individuals in roots was extremely low. The reason for this observation is unclear but it is not excluded that in presence of a low population of nematodes, root growth may be stimulated as a response to weak stress (hormesis phenomenon – Duke *et al.*, 2006). To the contrary, SDW of *C. spectabilis* was decreased in presence of nematodes. Similarly to the above, the reason for this is unclear. The stimulation of root growth may conversely impact shoot development, at least transiently. Duke *et al.* (2006) reported that under low elicitation (e.g. in presence of few nematodes), roots growth could be stimulated while no simultaneous effect is observed on SDW.

The number of juveniles, females and males as well as total number of nematodes was strongly decreased in the banana plants associated to AMF. Similar results were obtained *in vitro* on transformed roots of carrot in presence of *P. coffeae* (Elsen *et al.*, 2003) and whole banana plants (Koffi *et al.*, 2013a; Anene *et al.*, 2013) grown in presence of *R. similis* and in pots on tomato plants in presence of *M. incognita* (Vos *et al.*, 2012a). Several mechanisms have been proposed and summarized by Koffi *et al.* (2013a). In the present experiment, it is suggested that the early and heavy root colonization by the AMF prior to any contact with *R. similis* increased the ability of AMF to compete with the nematodes as reported in several studies (Koffi *et al.*, 2013a; Anene *et al.*, 2013). The development of a profuse extraradical mycelium surrounding the banana roots may further impact the movement of nematodes toward the roots as documented by Vos *et al.* (2012a). Recently Vos *et al.* (2012b) reported a systemic resistance/tolerance induced in AMF-colonized banana plants against migratory nematodes (*P. coffeae*) and sedentary nematodes (*M. incognita*). Although we did not investigate this mechanism, it was repeatedly reported in the literature as a major driver of plant resistance/tolerance to nematodes (Azcón-Aguilar and Barea, 1996; Elsen *et al.*, 2008; Vos *et al.*, 2012a, 2012b). Several studies also reported that the resistance/tolerance conferred by AMF against nematodes was due to the accumulation of phenolic compounds in colonized roots (Zhang *et al.*, 2013). Similarly, this possibility was not explored but could not be excluded.

C. spectabilis was reported in several studies to impact the population of nematodes within soil (Wang *et al.*, 2002). Halbrendt (1996) and Wang *et al.* (2002) demonstrated that the production of secondary metabolites (i.e. monocrotaline) by *Crotalaria* species were inhibitory to the migratory nematode *Xiphinema americanum*. This secondary metabolite was also reported to inhibit the hatching of eggs of *M. incognita* and induced jerking of juveniles (Thoden *et al.*, 2009). The inhibition of eggs and jerking of juveniles may conduct to an important decrease of juvenile's population. Wang and McSorley (2012) also suggested that *Crotalaria* species were able to enhance the population of natural enemies of plant parasitic nematodes, such as nematode trapping fungi or fungi feeding on eggs. Interestingly, the number of juveniles and the total number of nematodes in banana roots was decreased in the presence of *C. spectabilis*. This suggested either an effect on the population of nematodes in the surrounding soil (Wang *et al.*, 2002), decreasing thus their infection of roots, or an effect on the neighbor banana plants, via stimulation of their defense mechanisms.

The impact of AMF or *C. spectabilis* on the total population of nematodes resulted in a drastic decrease in the surface of necrotic roots. Interestingly, the combination of AMF and *C. spectabilis* reduced even more significantly the surface of necrotic tissues, suggesting a synergistic effect of their combination. Mechanisms such as competition or inhibitory effects of secondary metabolites within the soil surrounding the roots or competition or stimulation of host defenses within the roots may be involved.

CONCLUSION

In the present study, we demonstrated that the concomitant application of AMF and *C. spectabilis* is a thoughtful option to mitigate the impact of *R. similis* in bananas. The pre-mycorrhization (*in vitro* or *in vivo*) of bananas during the weaning phase, i.e. in absence of nematodes, and their further plantation in presence of *C. spectabilis* as intercrop may possibly impact the nematodes population within roots and thus reduce the doses or frequency of nematicides applications. This route may be part of an IPM strategy in banana plantation. It is, however, obvious that such strategy needs first to be evaluated under field conditions to appraise its real potential.

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CHAPTER 3

Root exudates of *Crotalaria spectabilis* colonized or not by *Rhizophagus irregularis* impacts viability and chemotaxis of the nematode *Radopholus similis*

Abla Anene and Stéphane Declerck (2017)

Research article in preparation for publication

Preface

In chapter 2, we investigated the combination of the AMF *R. irregularis* MUCL 41833 and the push pull plant *C. spectabilis* on the population density and damage of *R. similis* on banana plantlets. We demonstrated that the concomitant use of the AMF and *C. spectabilis* impacted all the developmental stages of the nematodes, decreasing their population and multiplication rate in banana roots more than the AMF or *C. spectabilis* alone. Roots necrosis index (RNI) was significantly reduced with the combination of AMF and *C. spectabilis* as compared to both treatments separately.

It has been amply demonstrated that AMF modify root exudates of colonized plants. Interestingly, *C. spectabilis* produces some metabolites that are known to impact nematodes. It is, however, unknown if AMF may modify exudation of *C. spectabilis* and if exudates of AMF-colonized *C. spectabilis* plantlets are more efficient against nematodes than exudates of non-colozined *C. spectabilis* plantlets.

In chapter 3, we compared the effects of root exudates of AMF-colonized and non-colonized *C. spectabilis* plantlets on the chemotaxis and viability of *R. similis*. Plantlets exudates were produced under hydroponic *in vitro* culture conditions to avoid any confounding effects with unwanted microbes. Nematodes movement were tested in presence of different combinations of exudates and their viability was evaluated in presence of different concentrations of root exudates.

Abstract

Crotalaria spectabilis is a mycotrophic push pull cover crop, which effects on the control of nematodes is related to the exudation of secondary metabolites. It is well known that AMF modify qualitatively and quantitatively the plant root exudates. However, it is unknown to what extend these modifications may affect the exudates of *C. spectabilis* and thus impact nematodes. Here, root exudates of AMF-colonized and non-colonized *C. spectabilis* plantlets and of non-colonized banana plantlets were evaluated on chemotaxis and viability of *R. similis*. For chemotaxis bio-assay, nematodes were placed in the middle of Petri plates and their direction of movement evaluated when confronted at opposite sides with different combinations of exudates. For viability, nematodes were plunged for 24, 48 and 72 h in increasing concentrations of exudates and their viability evaluated. Nematodes were more attracted by the exudates (particularly banana exudates) as compared to the controls (i.e. water). Curiously, they were more attracted by exudates of *C. spectabilis* with/without AMF when confronted to banana exudates. Finally, the presence of AMF did not influence the direction of nematodes, even if nematodes slightly preferred to move in the direction of exudates of AMF-colonized *C. spectabilis* when compared to banana exudates or controls. The viability of nematodes was markedly decreased after plunging in *C. spectabilis* exudates (particularly at 48 and 72 h) but recovered mobility after cleaning from the exudates. These results suggested that exudates of *C. spectabilis* have an attractive effect on nematodes which is not or only slightly increased in presence of AMF and that the exudates are nematostatic rather than nematocidal.

Keys words: *Crotalaria spectabilis*, arbuscular mycorrhizal fungi, hydroponic *in vitro* culture system, root exudates, *Radopholus similis*

INTRODUCTION

Push pull (or stimulo-deterrent diversion) farming systems are receiving an increasing interest to control pests for sustainable intensification of food production (Pickett *et al.*, 2014). For instance, the combination of a cash crop with a companion plant delivering secondary metabolites active against nematodes (e.g. *R. similis, M. arena*) has been recommended in banana production systems (Risède *et al.* 2010a, b) and is accessible to small hold farmers.

Several plants are considered as push pull crops (Pickett *et al.,* 2014). Among these are a number of species belonging to the *Crotalaria* genus, which include about 500 taxa of herbs and shrubs. For instance, *C. juncea*, *C. spectabilis* and *C. retusa*, which are grown as cover crops and/or green manure to maintain soil stability and fertility (Dorel *et al.*, 2013), are also poor or non-hosts for a large group of pests including nematodes (see section 2.4).

Crotalaria spectabilis is non-host for sedentary nematodes (e.g. *Meloidogyne incognita, Heterodera glycines*) (Rich and Rahi, 1995; Wang *et al.* 2002; Kushida *et al.*, 2003; Germani and Plenchette, 2004) and poor-host for some migratory nematodes (e.g. *Pratylenchus penetrans*) (Jasy and Koshy, 1994; Wang *et al.,* 2002; Thoden *et al.,* 2009; Wang and McSorley, 2012). Its repellent effect is probably related to the exudation of secondary metabolites (Pyrrolizidine alkaloid molecules) such as monocrotaline as demonstrated by Wang *et al.* (2002) and Thoden *et al.* (2009) against *M. incognita*.

Monocrotaline can be collected from the seeds or leaves (seed/leaf extracts) and from the roots (root exudates). For example, ground seeds of *C. spectabilis* incorporated into the soil at 2% of soil volume suppressed the whole population of the root-knot nematodes *M. javanica* and *M. incognita* (Rich and Rahi, 1995). In another study, L'Etang (2012) reported a nematostatic effect of root exudates of *C. spectabilis* on *M. arenaria*. However, nowadays most investigations were focused on sedentary nematodes and the effects of *Crotalaria* species on migratory nematodes are less documented.

Arbuscular mycorrhizal fungi are key organisms inhabiting the rhizosphere, forming obligate symbiotic associations with 74% of Angiosperm species (Brundrett, 2009). Their effects on soil structure (Barea *et al.*, 2002a) and plant nutrients uptake (Smith and Read, 2008) as well as on plant protection against abiotic (Plouznikoff *et al.*, 2016) and biotic (Whipps, 2004) stresses have been extensively reported. In particular, their effects on nematodes have been repeatedly demonstrated (Lax *et al.* 2011; Vos *et al.* 2012b) and for banana resulted in a marked decrease of the population of, for instance *R. similis*, as well as their multiplication rate and impact on roots (Elsen *et al.* 2008; Koffi *et al.* 2013a; Schouteden *et al.* 2015).

Interestingly, *Crotalaria* species are highly mycotrophic plants (Germani and Plenchette 2004). Recently, Anene and Declerck (2016) demonstrated that the concomitant use of *C. spectabilis* with the AMF *R. irregularis* MUCL 41833 markedly decreased the surface of banana roots damaged by the nematodes. The effects were even higher as compared to AMF or *C. spectabilis* alone, opening the door for a combined utilization of AMF and push pull plants in an integrated management of nematodes.

The mechanism by which AMF and *C. spectabilis* together decrease the pressure caused by nematodes remains poorly explored. It is well documented that AMF induce qualitative and quantitative changes in root exudates of colonized plants (Cordier *et al.* 1998; Norman and Hooker 2000; Tahat *et al.* 2008). Interestingly, in a chemotaxis bio-essay comparing exudates of non-mycorrhizal versus mycorrhizal roots of tomato, Vos *et al.* (2012c) observed a clear chemotactic response of *R. similis* moving away from the mycorrhizal root exudates. Therefore, it is tempting to speculate that AMF could similarly modify the exudation of *C. spectabilis* roots and hypothetically increase its efficacy in controlling nematodes.

The objective of the present study was to evaluate whether the root exudates of AMF-colonized *C. spectabilis* plants were more efficient against *R. similis* than the exudates of non-colonized plants. Therefore we monitored the chemotaxis and viability of nematodes in presence of exudates extracted from AMF-colonized and non-colonized roots of *C. spectabilis* as well as of banana roots used as control.

MATERIALS AND METHODS

Biological material

Seeds of *C. spectabilis* Roth (Wolfseeds, Brazil) and *M. truncatula* Gaertn. c.v. Jemalong strain A17 (SARDI, Australia) were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min, rinsed in sterilized (121 °C for 15 min) deionized water and further germinated in 90 mm Petri plates filled with 35 ml of the Modified Strullu-Romand (MSR) medium (Declerck *et al.* 1998) solidified with 3 g L^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA) following the method of Cranenbrouck *et al.* (2005). The Petri plates were incubated in the dark at 27°C for 4 days before exposure to light for 2 days until use.

Tissue-cultured banana plantlets (*M. acuminata* Colla c.v. Grande Naine, clone CV902) were provided by VITROPIC SA (Montpellier, France). The plant material was proliferated, regenerated and rooted on the Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel, and with pH adjusted between 6.12 and 6.15 before sterilization (Banerjee and De Langhe 1985). The plantlets were incubated in a growth chamber at 27/25 °C (day/night) with a photoperiod of 16 h day^{-1}, a relative humidity (RH) of 70% and a photosynthetic photon flux (PPF) of 300 $\mu mol\ m^{-2}s^{-1}$.

A strain of *R. irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 was supplied by GINCO on Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 grown in 90 mm Petri plates on the MSR medium. The Petri plates were maintained in the dark in an inverted position at 27 °C until thousands of spores were obtained.

A highly virulent strain of *R. similis* (Cobb) Thorne (Anene and Declerck, 2016) originating from Uganda was provided by the Nematology laboratory of K.U. Leuven (Belgium). The nematodes (juveniles and adults) were maintained under aseptic conditions on carrot discs (Pinochet *et al.* 1995) at 28°C in the dark.

Experimental set up

Pre-mycorhization of *C. spectabilis* seedling

Crotalaria spectabilis and banana plantlets were colonized using the Mycelium Donor Plant (MDP) *in vitro* culture system (see Voets *et al.*, 2005). Briefly, bi-compartmented Petri plates were constructed by introducing the lid of a small Petri plate (named the root compartment – RC) in the bottom of a large Petri plate (named the hyphal compartment – HC) (see for details Koffi *et al.* 2013a). Both compartments were filled with MSR medium without sucrose and vitamins (called MSRss throughout the text) (20 ml and 80 ml in the RC and HC, respectively) as described by Anene *et al.* (2013). One week-old *in vitro* produced *M. truncatula* seedling was associated to the AMF in the RC and incubated in a growth chamber (27/22 °C day/night, a photoperiod of 16 h day^{-1}, a RH of 70% and a PPF of 300 µmol m^{-2}s^{-1}). After 4 weeks, the AMF densely colonized the RC and crossed the plastic barrier separating the RC from the HC to develop profusely in the HC. At that time, five *C. spectabilis* seedlings were introduced in the HC with their roots plated on the MSRss medium in contact with the extraradical mycelium (ERM) of the fungus and shoots extending outside the plates via holes cautiously plastered to avoid contaminations. An identical number of plantlets was placed in similar systems as above but without AMF (i.e. the controls). The seedlings were incubated for 3 weeks under the same conditions as above. Fifty ml of MSRss medium was added weekly in the RC and HC of each system. After 3 weeks, root colonization was evaluated on 6 randomly chosen plantlets by the method of Mc Gonigle *et al.* (1990). The % root colonization (%RC), % arbuscules (%A) and % spore/vesicles (%V) was 100, 30 ± 45 and 76 ±16, respectively.

Hydroponic cultivation system set up

A hydroponic cultivation system was used to grow the AMF-colonized and non-colonized plantlets of *C. spectabilis* and non-colonized banana plantlets for extraction of root exudates. Briefly, a large Petri plate (145 mm diam.) was filled with 90 ml of liquid MSRss medium. A hole of ± 2 mm diameter was made in the base and lid of the Petri plate just above the surface of the liquid medium. One pre-mycorrhized plantlet of *C. spectabilis* was gently uprooted from the *in vitro* culture system described above and the remaining solid medium attached to the roots was carefully removed with sterile forceps. The plantlet was then introduced into the Petri plate with its roots bathing in the liquid MSRss medium and shoot extending outside the Petri plate via the hole.

The same procedure was applied with the non-mycorrhized *C. spectabilis* and with the banana plantlets. Twenty four replicates were set up per treatment. The liquid medium in each system was replaced every 10 days by fresh liquid medium via a hole of 14 mm diam. made on the lid of the Petri plate and surmounted with a Falcon tube cut in the middle and glued with hot melt glue (Metabo®) (Fig. 22a). The systems were maintained during 6 weeks (equal to 2 months old plantlets, age of optimum root exudates production of *C. spectabilis* (L'Etang 2012)) under the same conditions as above. Root colonization by AMF was evaluated on 6 randomly selected *C. spectabilis* plantlets (Mc Gonigle *et al.* 1990) before root exudates collection.

Root exudates collection

Root exudates were collected following a procedure adapted from the method developed by Tran *et al.* (2004). Briefly, after culture in the liquid MSRss medium, the plantlets were removed and their roots gently washed with deionized sterilized water. The plantlets were subsequently placed in a sterile Erlenmeyer Pyrex® Glass flask containing 50 ml of sterilized (121 °C for 15 min) deionized water. The water was maintained at 45 °C for 8 h and then at 28 °C for 16 h by plunging the flasks in a water bath. Only the roots were in contact with the water. After this period, the solution was collected and filtered with acrodisc® syringe filters (2 µm) (Sigma- Belgium) and further freeze-dried (Labconco®- Belgium) before storage at -20°C.

The exudates solution was prepared by dissolving 1 mg.ml^{-1} of the freeze-dried powder of mycorrhized or non-mycorrhized *C. spectabilis* plantlets or of banana plantlets in sterilized (121°C for 15 min) deionized water.

Impact of root exudates on nematodes chemotaxis

A bio-assay was set up to investigate the role of exudates on nematode chemotaxis. The protocol used was adapted from Vos (2012a). Briefly, small Petri plates (6 cm diam.) were filled with 10 ml of MSRss medium solidified with 2.4 g L^{-1} phytagel. Each Petri plate was divided in two equal compartments. A line of 10 x 2 µl droplets of exudates solution (i.e. 20 µl) was added at equal distance (i.e. 1.5 cm) at both sides of the middle of the Petri plate. The Petri plate was kept under the laminar flow for 2 h to allow the roots exudate to diffuse in the medium. Ten juveniles and adults *R. similis* nematodes were subsequently placed from bottom to top in the middle of the Petri plate and chemotaxis monitored starting 1 h after plating (Fig 21). Different combinations were tested: root exudates of mycorrhized *C. spectabilis* versus root exudates of non-mycorrhized *C. spectabilis* ($\text{Exud}^{\text{Cs+AMF}} \neq \text{Exud}^{\text{Cs-AMF}}$); root exudates of mycorrhized *C. spectabilis* versus sterilized deionized water ($\text{Exud}^{\text{Cs+AMF}} \neq$ Control); root exudates of non-mycorrhizal *C. spectabilis* versus sterilized deionized water ($\text{Exud}^{\text{Cs-AMF}} \neq$ Control); root exudates of mycorrhizal *C. spectabilis* versus root exudates of banana ($\text{Exud}^{\text{Cs+AMF}} \neq \text{Exud}^{\text{Ban}}$); root exudates of non-mycorrhizal *C. spectabilis* versus root exudates of banana ($\text{Exud}^{\text{Cs-AMF}} \neq$

Exud^{Ban}); root exudates of banana versus sterilized deionized water ($\text{Exud}^{\text{Ban}} \neq$ Control) and sterilized deionized water versus sterilized deionized water (Control ≠ Control). Each treatment consisted of 10 replicates.

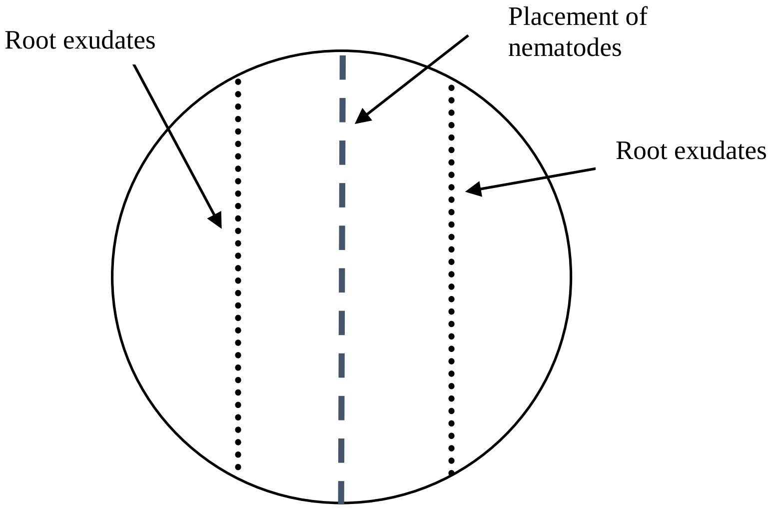


Figure 21: Experimental design to monitor the chemotaxis of *R. similis* in presence of various combinations of root exudates of AMF-colonized or non-colonized *C. spectabilis* or banana plantlets.

The Petri plate was divided in 2 equal compartments. Exudates (10 X 2 µl) were added to the MSRss medium in a line, 1.5 cm away from the middle of the Petri plate, in each direction and left to diffuse for 2 h. Ten juveniles and adults' nematodes were subsequently placed in the middle of the Petri plate and movement monitored starting 1 h after plating.

Impact of root exudates on nematodes viability

A bio-assay was set up to evaluate the viability (completely immobile) of nematodes in presence of increasing concentrations of exudates following the protocol developed by Vos *et al.* (2012b). Root exudates solutions were prepared by dissolving 0.1 mg ml^{-1} (C_1), 1 mg ml^{-1} (C_2) and 10 mg ml^{-1} (C_3) freeze dried powder of AMF-colonized and non-colonized *C. spectabilis* root exudates in sterilized (121°C for 15 min) deionized water. Glass vials (Pyrex®) of 6 ml were filled with 1 ml of a nematode suspension (approximately 400 *R. similis* juveniles and adults) and 1 ml of each root exudates solution. Glass Vials with 1 ml of a nematode suspension and 1 mg ml^{-1} freeze dried powder of banana root exudates or 1 ml of sterilized deionized water alone were used as controls. Glass vials were stored in the dark at room temperature (20 ± 3 °C) during the whole time of the incubation. After 24, 48 and 72 h incubation, the glass vials were shaken for 30 s. Two hundred fifteen µl of the nematodes solution was pipetted into a hollow slide containing 2 ml of sterilized (121°C for 15 min) deionized water and their viability monitored under a compound microscope (Olympus BH2, Olympus Optical, and GmbH, Germany) at 20 x magnification. Each treatment was repeated 6 times. The nematodes (mobile and immobile) were finally transferred into a Petri plate containing 5 ml of fresh sterilized (121°C for 15 min) deionized water and their viability evaluated after 24 h to assess the nematostatic/nematicidal effect of the root exudates.

Statistical analysis

All the statistics were conducted with the SAS Enterprise Guide. Data for nematodes chemotaxis were analyzed by Chi Square test (χ^2) with p-values corrected by Bonferroni, while nematodes viability were analyzed by Kruskal-Wallis test ($P<0.05$). Prior to statistical analyses, nematodes numbers were log (x+1) transformed to reduce data variance.

RESULTS

AMF-colonized and non-colonized plantlets of *C. spectabilis* and banana plantlets were placed for 6 weeks in liquid MSRss medium with their shoots extending outside the Petri plate and roots bathing in the liquid MSRss medium before transfer to flasks for production of exudates. All the plantlets grew well, producing a dense root mat (Fig. 22a) and novel leaves. Hyphae emerged from the roots of the AMF-colonized *C. spectabilis* plantlets and developed profusely in the liquid MSRss medium producing a dense extraradical mycelium bearing hundreds of spores (Fig. 22b). Total root colonization, estimated at 6 weeks, was 100% with 89 ± 7% spore/vesicles and only a few arbuscules.

Figure 22: *In vitro* hydroponic cultivation system with AMF-colonized *C. spectabilis* plantlets.
(a) Plant shoot developing in open air conditions and roots (R) in liquid MSRss medium. Scale bar = 25 mm. (b) A close up of the root system with hundreds of extra-radical spores (S) and hyphae (H) extending from the roots. Scale bar = 500 µm.

Effects of root exudates on nematodes movement

The direction of movement of the nematodes was monitored in Petri plates in presence of different combinations of root exudates (Fig 23). In the $\text{Exud}^{\text{Cs+AMF}} \neq \text{Exud}^{\text{Cs-AMF}}$ treatment, 42% of the nematodes remained in the middle of the Petri plates, while 29% moved to one or the other side with no significant differences between both ($p = 0.5535$).

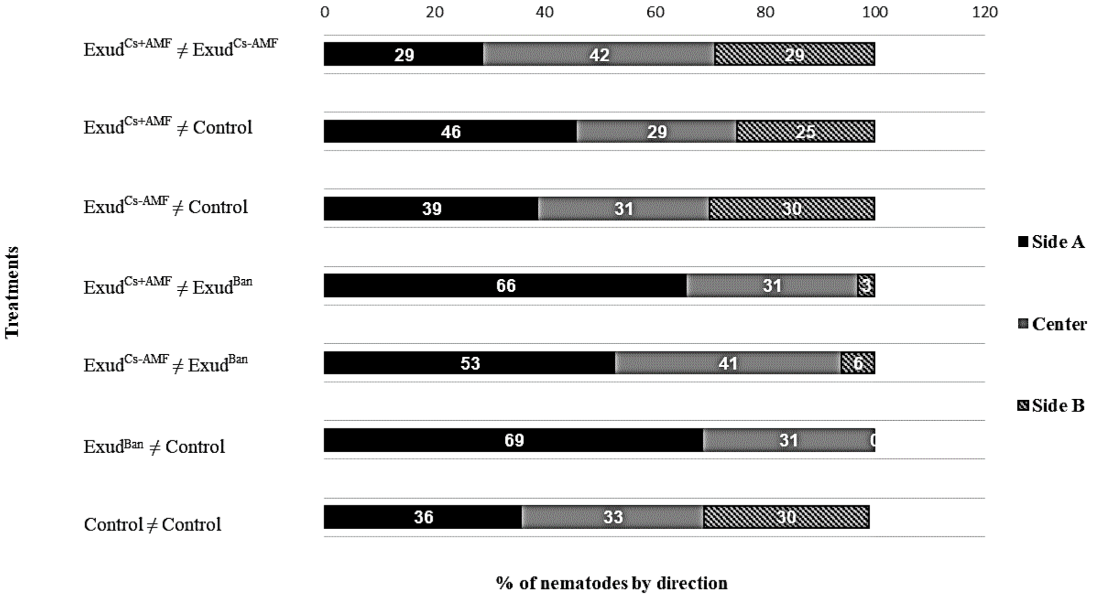
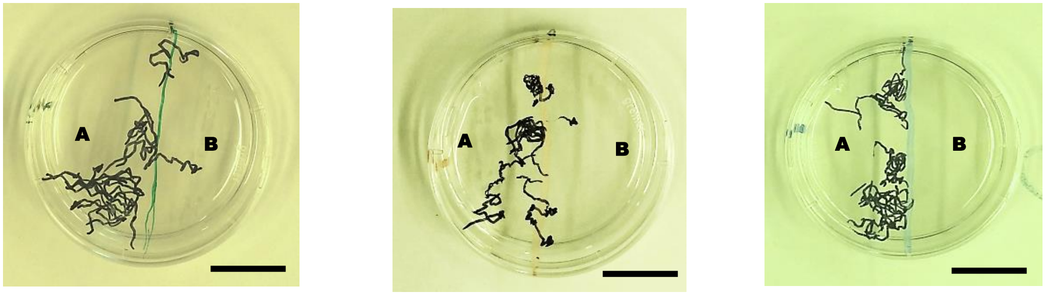


Figure 23: ***In vitro* evaluation of the orientation of nematodes as a function of the exudates present in the culture medium**.

Side A: % of nematodes moving in the direction of the root exudates on the left side of the middle of the Petri plate. Side B: % of nematodes moving in the direction of the root exudates on the right side of the middle of the Petri plate. Center: % of nematodes remaining in the center of the Petri plate.

In the $\text{Exud}^{\text{Cs+AMF}} \neq$ Control and $\text{Exud}^{\text{Cs-AMF}} \neq$ Control treatments, 31% and 29%, respectively, of the nematodes remained in the middle of the Petri plates, while 46% and 39% moved in the direction of the root exudates of *C. spectabilis* colonized or not by the AMF, respectively. However, difference was only significant for the $\text{ExudCs}^{\text{+AMF}} \neq$ Control treatment ($p = 0.0239$). In the $\text{Exud}^{\text{Cs+AMF}} \neq \text{Exud}^{\text{Ban}}$ and $\text{Exud}^{\text{Cs-AMF}} \neq \text{Exud}^{\text{Ban}}$ treatments, a significantly ($p< 0.001$) higher number of nematodes (66% and 53%, respectively) moved in the direction of roots exudate of *C. spectabilis* as compared to the banana root exudates (3% and 6%, respectively)

(Fig. 24 a and b). Conversely, in the Exud^{Ban} $\neq$ Control treatment, the nematodes moved preferably (i.e. 69 %) in the direction of the Exud^{Ban} ($p < 0.001$) (Fig. 24c). In the Control $\neq$ Control treatment, no significant differences were noticed in nematodes movement in one or another direction.



a b c

Figure 24: *In vitro* chemotaxis bio-assay and visualization of nematode movement on the medium.

Drawing of movement was done 1h after inoculation with 10 juveniles and females nematodes of *Radopholus similis* plated in the middle of the Petri plate. At both sides (left (A) and right (B)), 1.5 cm away from the middle of the Petri plates, 10 x 2µl droplets of exudates were poured in a line. The treatments are a = Exud^{Cs+AMF} $\neq$ Exud^{Ban}; b = Exud^{Cs-AMF} $\neq$ Exud^{Ban}; c = Exud^{Ban} $\neq$ Control. Nematodes movement was monitored and preferentially moved to the side containing exudates of *C. spectabilis* colonized or not by *R. irregularis* (respectively a and b). However, in absence of *C. spectabilis* (case c), the nematodes moved preferentially to banana root exudates. Scale bar = 20 mm.

Impact of root exudates on nematodes viability

Whatever the time of incubation (i.e. 24, 48 and 72h), almost all the nematodes were mobile in the Exud^{Ban} and deionized water controls (99.5% and 100%, respectively). At C$_1$, the percentage of immobile nematodes in the Exud^{Cs+AMF} and Exud^{Cs-AMF} treatments was below 11%, whatever the time of incubation, and did not differ between both treatments ($p = 0.739$; Fig. 25). At C$_2$, the % of immobile nematodes was significantly higher in the Exud^{Cs-AMF} treatment as compared to the Exud^{Cs+AMF} treatment at 24 h ($p < 0.05$). This difference disappeared at 48 and 72h ($p = 0.797$ and 0.618, respectively) (Fig 5) with % of immobile nematodes always above 80%. Interestingly, at 24 h, the % of immobile nematodes in the Exud^{Cs+AMF} was close to 20%, while it increased significantly ($p = 0.002$) to 100% and 80% at 48 and 72 h, respectively (Fig 25). A similar observation was made at C_3. At 24 h, the % of immobile nematodes was significantly lower in the Exud^{Cs+AMF} as compared to the Exud^{Cs-AMF} treatment ($p < 0.05$), while no difference was noticed at 48 and 72 h (Fig 25). At 24 h, the % immobile nematodes in the Exud^{Cs+AMF} was below 20%, while it was 100% at 48 and 72 h. At C_2 and C_3, the % immobile nematodes in the Exud^{Cs-AMF} was almost 100 % (Fig 25).

The nematodes incubated in the different concentrations of exudates during 24, 48 and 72h were rinsed and placed in sterilized deionized water, and their viability evaluated after 24 h. Whatever the treatment and duration of incubation, nearly all of the nematodes were mobile (i.e. 100 % after 24 and 48 h incubation for the Exud^{Cs+AMF} and Exud^{Cs-AMF} treatments and 98% for the Exud^{Cs+AMF} and 96% for the Exud^{Cs-AMF} treatment after 72 h incubation) with no significant differences noticed between the treatments.

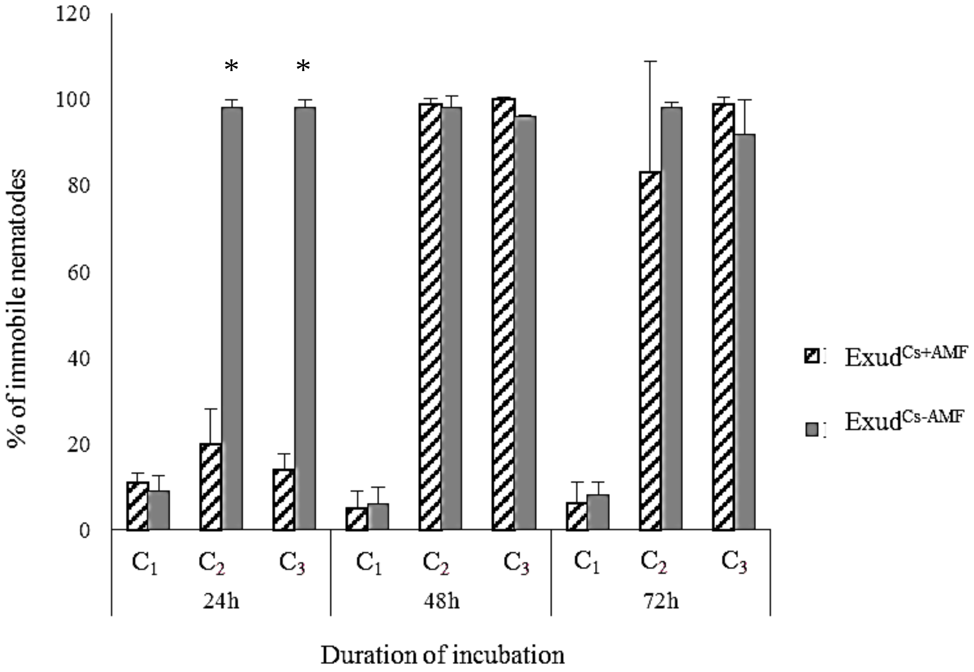


Figure 25: Percentage of immobile nematodes following incubation in different concentrations of exudates from AMF-colonized and non-colonized roots of *C. spectabilis*.

Nematodes were incubated at 3 different concentrations of root exudates (C1 = 0.1 mg ml^{-1}; C2= 1 mg ml^{-1}; C3 = 10 mg ml^{-1}) during 24, 48 and 72 h. Their viability (i.e. the number immobile and mobile individuals) was subsequently monitored in hollow slides. *: % of immobile nematodes is significantly difference between Exud^{Cs+AMF} and Exud^{Cs-AMF} the first 24h with C1 and C2.

DISCUSSION

Root colonization by AMF induces qualitative and quantitative changes in plant exudates (Barea *et al.* 2002b). However, it is largely unknown if root colonization of a push pull plant (i.e. *C. spectabilis*) similarly affects the production of exudates and thus impacts (i.e. improves or inhibits) its effects on nematode chemotaxis and viability. To answer this question, an *in* *vitro* bio-assay was set up comparing the effects of root exudates of *C. spectabilis* colonized or not by the AMF *R. irregularis* MUCL 41833 on chemotaxis and viability of *R. similis*. Root exudates of non AMF-colonized banana plantlets were also included in the test as positive control.

All the plantlets, colonized or not by the AMF, grew well with novel roots and leaves produced. An important root colonization by the fungus was also observed. The nematodes preferentially moved towards exudates produced by *C. spectabilis* or banana when they had the choice between exudates and absence of exudates. Moreover, in presence of the exudates of the two plants, the direction of the nematodes was preferentially in the direction of the exudates of *C. spectabilis*. The AMF did not visibly impact the direction of movement of the nematodes. The viability of nematodes was significantly impacted by the exudates of *C. spectabilis*. However, this effect was nematostatic and not nematocidal.

Crotalaria spectabilis plantlets were colonized by the AMF in the MDP *in vitro* culture system (Voets *et al.* 2005) filled with MSRss medium (Anene *et al.*, 2013). Plantlets were heavily colonized (%RC, %A and %V was 100, 30 ± 45 and 76 ±16, respectively) within 3 weeks and subsequently transferred in liquid MSR medium, first in Petri plates (during 6 weeks) for development and adaptation and secondly in flasks (at 45 °C for 8 h and then at 28 °C for 16 h) for exudates production, using the method of Tran *et al.* (2004). The plantlets developed well in the liquid MSRss medium. *Crotalaria spectabilis* as well as banana plantlets doubled their size in a period of 6 weeks (data not shown). Similarly, root colonization of *C. spectabilis* plantlets was high with value of total root colonization reaching 100%, 6 weeks after transfer in the liquid MSRss medium. A profuse extraradical mycelium bearing hundreds of spores was noticed (data not shown).

When comparing to the control (i.e. sterilized deionized water), nematodes moved preferentially in the direction of exudates. This was particularly marked with the exudates of bananas and to a lesser extend with the exudates of *C. spectabilis* colonized or not by the AMF. This suggested that banana exudates were more attractive for nematodes than *C. spectabilis* exudates and confirmed the susceptibility of cultivar Grande Naine to *R. similis*. However, when the exudates of both plants were opposed, the nematodes moved preferentially in the direction of *C. spectabilis* exudates (in presence as well as in absence of the AMF). This was particularly curious since several studies have reported that migratory nematodes such as *R. similis* were unable to multiply on *C. spectabilis* (Jasy and Koshy 1994; Wang *et al.* 2002; Wang and McSorley 2012). This poor host effect of *C. spectabilis* was also observed by Kushida *et al.* (2003) with soybean cyst nematode (*Heterodera glycines*) and by Germani and Plenchette (2004) with root-rot nematodes (*M. javanica* and *M. incognita*). The attraction of *C. spectabilis* root exudates on *R. similis* remains thus to be understood. It is often proposed that some molecules (monocrotaline) are repellent to nematodes (Thoden *et al.* 2009), although it is not excluded that other chemicals may be attractant to nematodes (Reynolds *et al.*, 2010), possibly at concentrations that mask the effects of monocrotaline or that the nutrients composition (e.g. carbon content) of *C. spectabilis* exudates may be more attractive than the nutrient composition of banana root exudates. Trap crops, which are plants that support nematode invasion but do not permit the completion of their life cycle has also been suggested in nematodes management (Viaene *et al.*, 2013). Interestingly, Kushida *et al.* (2003) demonstrated the trap effect of *C. spectabilis* and *C. juncea* on the soybean cyst nematodes *H. glycines*. These authors reported that the two *Crotalaria* species stimulated eggs hatching and had the same number of juveniles penetrating the host plant. Interestingly, the juveniles that penetrated into *Crotalaria* roots did not complete their life cycle, resulting in a significant decrease in nematodes population. Therefore it is not excluded that the attraction effect of *C. spectabilis* exudates observed in our study on *R. similis* may be related to a “trap” effect of this crop.

Root colonization by AMF did not influence the direction of nematodes. Indeed, when root exudate of colonized *C. spectabilis* were opposed to root exudates of non-colonized *C. spectabilis*, a similar number of nematodes moved in both directions. Interestingly, AMF-colonized *C. spectabilis* were more attractive to nematodes than non-colonized *C. spectabilis* root exudates when opposed to banana exudates. Therefore, it cannot be totally excluded that AMF might slight modify root exudates composition and influence nematode movement. This was demonstrated by L'Etang (2012). These authors analyzed root exudates of AMF-colonized and non-colonized *C. spectabilis* plantlets and noticed that the colonized plantlets had a higher percentage of alkaloids and phenolic compounds than non-colonized *C. spectabilis* plantlets. The different composition may somehow modify quantitatively or qualitatively the composition of exudates, slightly influencing the mobility of nematodes.

Root exudates of *C. spectabilis* and bananas differently impacted nematodes viability. In presence of banana exudates or deionized water (i.e. the control) and whatever the duration of incubation (24, 48 or 72 h), almost 100% of the nematodes were mobile. Conversely, the incubation in exudates of *C. spectabilits* strongly impacted the nematodes mobility. The impact was already slightly visible after 24 h incubation and was maximum after 48 and 72 h of incubation whatever the concentration of exudates. At both times, between 80 and 100% of the nematodes were immobile. These results suggested that exudates had a paralyzing effect on nematodes. L'Etang (2012) reported a similar effect when *R. similis* and *M. arenaria* were incubated for 48 h in root exudates of *C. spectabilis* plantlets colonized by *G. intraradices*. Interestingly, Osei *et al.* (2010) noticed that monocrotaline, a potentially nematotoxic compound identified in *C. spectabilis,* inhibited the movement of juveniles of *Meloidogyne* species. The present results thus confirmed the probable presence of monocrotaline and/or other compounds in the roots of *C. spectabilis* inhibiting the movement of nematodes. Exudates from roots colonized by AMF did not differently impact viability of nematodes suggesting the absence or at least the non-discernable effect of root exudates of AMF-colonized plantlets. Interestingly, at 24 h incubation in the exudates, the mean % of immobile nematodes was significantly lower in AMF-colonized *C. spectabilis* plantlets at concentration 1 mg ml^{-1} and 10 mg ml^{-1}. The reason for this is unknown, although it could be hypothesized that inhibitory molecules (e.g. monocrotaline) were present at a lower concentration and were thus less detrimental, in the short term, to nematodes viability.

Exudates concentrations had a strong impact on nematode viability. Indeed, at 0.1 mg ml^{-1}, the % of immobile nematodes was significantly lower as compared to 1 mg ml^{-1} or 10 mg ml^{-1} whatever the duration of incubation. This clearly demonstrated that the concentration of inhibitory molecules is of high importance and that below a defined concentration, no noticeable effect was observed. Interestingly, whatever the concentration of exudates and duration of incubation, the nematodes (immobile and mobile) cleaned from the exudates and placed in sterilized deionized water, totally recovered their mobility. This suggested that the inhibitory molecules had a nematostatic rather than nematocidal effect. This was similarly observed by L'Etang (2012) with *R. similis* and *M. arenaria* incubated during 48 h in root exudates of AMF-colonized *C. spectabilis* plantlets.

As a conclusion, root exudates of *C. spectabilis* were attractive to *R. similis*, keeping them away from banana exudates and thus potentially from banana plants. This suggested the potential role of *C. spectabilis* as "trap crop" against the migratory nematodes *R. similis* as it was earlier reported with sedentary nematodes belonging to *Meloidogyne sp*.. The influence of AMF on the exudates could not been demonstrated nor excluded. Therefore their direct impact on nematodes outside the roots via the modification of plant exudates remains questionable. Finally, the exudates of *C. spectabilis* were nematostatic and not nematocidal at the concentrations tested. It is obvious that in soil, the exudates may be more diluted, therefore only exerting nematostatic effects on *R. similis* in the close vicinity of roots.

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VI. GENERAL DISCUSSION

VI. GENERAL DISCUSSION

The undesired side-effects of nematicides on farmer's health and environment have encouraged the development of alternative measures to control nematodes in banana plantation such as fallow, paring and hot water treatment of the corms, the use of resistant cultivars and the planting of *in vitro* produced plantlets (Quénehervé, 2009). In the last few years, an increasing attention has also been devoted to the potentials offered by bio-control organisms to decrease the pressure caused by nematodes (e.g. Siddiqui and Mahmood, 1996; 1999; Duponnois *et al.*, 1998; Nega, 2014).

Among the bio-control organisms that are nowadays frequently considered against pests and diseases are the AMF. These soil inhabitants form symbiotic associations with an estimate of 74% of angiosperms (Brundrett *et al.*, 2009) and are present in almost all ecosystems. They have been reported to improve plant growth and increase plant resistance/tolerance against abiotic and biotic stresses (Smith and Read, 2008; Pozo *et al.*, 2013). In particular, their effects on decreasing the population of nematodes within roots have been repeatedly reported in studies conducted in pots (Vos *et al.*, 2012a) or *in vitro* on excised transgenic roots (Elsen *et al.*, 2003). This later method present two major advantages which are (1) the possibility to follow non-destructively the development of the nematodes and fungi and their interactions outside the roots and (2) the absence of any undesirable microbial contaminant that could interfere with both organisms and the roots. However, the absence of a true source-sink (i.e. the shoot) also present some drawbacks which are the absence of photosynthetic tissues and the use of transgenic roots. Therefore, a novel *in vitro* cultivation system was developed by Voets *et al.* (2005) based on the association of autotrophic micropropagated plantlets with AMF. This system was adapted by Koffi *et al.* (2009) to banana plantlets, offering wide perspectives to investigate, under strict controlled conditions, the effects of AMF on increasing the resistance/tolerance/tolerance of bananas against phytopathogenic nematodes.

In parallel to the application of biocontrol microorganisms is the use of push pull plants such as *Crotalaria* species (Hassanali *et al.*, 2008; ICIPE, 2011). These leguminous plants are grown as cover crops to control weeds (Wang and McSorley, 2012) and to provide green manure (Wang *et al.*, 2002). Interestingly, they are non-host for root-knot nematodes (e.g. *M. incognita*) (Wang *et al.*, 2002), poor-hosts for some migratory nematodes (e.g. *P. penetrans*) (Wang *et al.*, 2002; Thoden *et al.*, 2009) and are highly mycotrophic (Germani and Plenchette, 2004). Some *Crotalaria* species (e.g. *C. spectabilis*) have been recommended as trap crops in association with cash crops, to attract nematodes such as *Meloidogyne sp.*, *H. glycines* into their roots, further disrupting their life cycle (Kushida *et al.*, 2003; Viaene *et al.*, 2013). These plants, as well as AMF, have been reported to partially control nematodes in numerous studies (Wang *et al*, 2002; Germani and Plenchette 2004; Vos *et al.*, 2012a; Koffi *et al.*, 2013a). However, their concomitantly application has never been considered.

Within this thesis, we first investigated the impact of the AMF *R irregularis* MUCL 41833 alone (**chapter 1**) or in combination with the push pull plant *C. spectabilis* (**chapter 2**) on the population and incidence of *R. similis* in banana. Two culture systems were used: the *in vitro* culture system developed by Voets *et al.* (2005) and adapted by Koffi *et al.* (2009) for banana (**chapter 1**) and a split-pot greenhouse culture system (**chapter 2**).

The *in vitro* culture system (**chapter 1**) consisted of a banana plantlet (shoot + roots) grown *in vitro* on the modified Strullu-Romand (Declerck *et al.*, 1998) medium without sugar and vitamins. The banana plantlets were pre-mycorrhized in the mycelium donor plant (MDP) *in vitro* culture system (Voet *et al.*, 2005), transferred to the half-closed arbuscular mycorrhizal plant (H-AMP) *in vitro* culture system (Koffi *et al.*, 2009) and inoculated with nematodes (see Figure 14 in the materials and method section).

The split-pot greenhouse culture system (**chapter 2**) consisted of a bi-compartmented pot containing two plants (banana and *C. spectabilis*). The shoot parts and the roots of both plants were physically separated aboveground by a fixed talpa net and below-ground by a pocket of nylon mesh (30 µm) in which the root system of *C. spectabilis* was placed. The banana plantlets were pre-mycorrhized in the MDP *in vitro* culture system (as above) then transferred in the bi-compartmented microcosm system, while the seedlings of *C. spectabilis* were grown in a pot before their transfer to the system. The nematodes were subsequently inoculated (see Figure 18 in the materials and method section).

In both systems, the AMF and nematodes were able to complete their life cycle in the presence of banana plantlets. In the *in vitro* culture system, the population of nematodes was reduced by half in the roots of the colonized banana plantlets as compared to the non-colonized plantlets. This was particularly marked on the number of juveniles and adults, supporting earlier findings in greenhouse (Vos *et al.*, 2012a) and with *in vitro* transformed roots organs of carrot (Elsen *et al.*, 2001a, 2003). Conversely, the presence of nematodes did not influence root colonization by the AMF (**chapter 1**).

There was no significant difference in shoot dry weight between colonized and non-colonized banana plantlets. However it was obvious that the Petri plates used in the *in vitro* culture system were limited in volume for the growth of the plantlets, thus may be not adequate to evaluate the AMF impact on plant growth.

In the bi-compartmented split-pot greenhouse culture system, the banana plantlets grew well and the root systems were heavily colonized by the AMF without being impacted by the nematodes. Banana biomass was increased in presence of *C. spectabilis* and AMF, in absence as well as in presence of the nematodes. The multiplication rate of *R. similis* decreased almost three times in the AMF-colonized banana plantlets as compared to the control (banana without AMF or *C. spectabilis*). The number of juveniles, females and males as well as total number of nematodes was strongly decreased in the banana plantlets associated to AMF with a lower multiplication rate (respectively 106.7 ± 10 and 280.3 ± 82) as compared to the control. The root necrosis index (RNI) was also decreased by 3 in presence of the AMF or by 1.8 in presence of *C. spectabilis*. In presence of both organisms in combination, the RNI was decreased by 3.6 as compared to the control (**chapter 2**).

The results obtained *in vitro* and within pots, clearly demonstrated the effects of AMF on decreasing the population of nematodes within roots and on reducing the necrosis on roots. This corroborates earlier results obtained with *P. coffeae* on *in vitro* transformed roots of carrot (Elsen *et al.*, 2003), *R. similis* on whole plants of banana grown *in vitro* (Koffi *et al.*, 2013a) and *M. incognita* in tomato plants grown in pots (Vos *et al.*, 2012a). Numerous mechanisms have been proposed (see review by Schouteden *et al.*, 2015) to explain these results.

The early and heavy root colonization by the AMF prior to any contact with *R. similis* was most often associated to a decrease in the incidence of nematodes on roots (Pozo *et al.*, 2009; Jung *et al.*, 2012; Koffi *et al.*, 2013a). Indeed, competition for space could be involved in AMF-nematodes interactions since they both reside inside the roots (Jung *et al.*, 2012, Viaene *et al.*, 2013). This was not evaluated in our study since the plantlets were always colonized prior to infestation by the nematodes, but pre-colonization, either *in vitro* (chapter I) or in pots (chapter II) clearly supported the strength of pre-mycorhization of the plantlets. Negative effects due to space limitation within roots has also been suggested as a mechanism on nematode control. Indeed, arbuscules exclusively form in the cortex, where migratory endoparasitic nematodes feed. Our results also supported this mechanism. In both experiments, a high percentage of arbuscules was observed in the banana plants grown in presence of nematodes with a concomitant decrease in nematode population (**chapter 1 and 2**).

Competition for nutrients uptake has also been reported in the literature as a possible mechanism of nematode control (Jung *et al.*, 2012). However this mechanism could not be ascertained in our study with the H-AMP *in vitro* culture system. Indeed, there was no significant difference in roots fresh weight and shoot dry weight between colonized banana and non-colonized banana plants (**chapter 1**). Conversely, the shoot dry weight was significantly increased in the AMF plants in the split-pot system (**chapter 2**). The absence of growth effect *in vitro* could be attributed to the limited mineral resources in the Petri plates, which was not the case in the split-pot system.

Another mechanisms, often reported in the literature, is the induced resistance/tolerance acquired by AMF-colonized plantlets. Vos *et al.* (2012b) using a split-root experimental set-up with tomato in which the root system of the plant was divided over two physically separated compartments with one side pre-inoculated with *G. mosseae*, demonstrated that *M. incognita* or *P. penetrans* infection in the other root compartment was significantly reduced, resulting in a systemic induced resistance/tolerance. Systemic suppression of nematodes infection in mycorrhizal roots has also been reported by Cordier *et al.* (1998), Pozo *et al.* (2002) and Elsen *et al.* (2008). This mechanism was not investigated in our thesis and should be considered in the near future. Indeed, the *in vitro* cultivation system even though limited in volume (and thus duration of experiment) is adapted to study the molecular mechanisms of biocontrol in the early stages of interaction as demonstrated by Gallou *et al.* (2011, 2012).

In the split-pot microcosm system joining bananas plants with the push pull plant *C. spectabilis*, we also observed that nematodes population was decreased as compared to the control. This corroborated earlier studies by Wang *et al.* (2002), Kushida *et al.* (2003), Germani and Plenchette (2004), Thoden *et al.* (2009). These authors mostly demonstrated that *C. spectabilis* inhibited eggs hatching and are repellent to migratory nematodes. A drastic decrease in nematodes multiplication rate and in root necrosis index was also noticed when *C. spectabilis* was associated to AMF in the split-pot system suggesting a potential synergism between the push pull plant and the fungus.

One mechanism proposed for the biocontrol by push pull plants is the production of secondary metabolites that are inhibitory to nematodes. For instance, Thoden *et al.* (2009) reported that *C. spectabilis* produce secondary metabolites (i.e. monocrotaline) that are inhibitory to the movement of migratory nematodes (Thoden *et al.*, 2009). Interestingly, it is well documented that AMF induce qualitative and quantitative changes in root exudates of colonized plants (Cordier *et al.*, 1998; Norman and Hooker, 2000; Tahat *et al.*, 2008). Therefore it is not excluded that AMF may modify qualitatively or quantitatively the exudates of *C. spectabilis* upon root colonization and potentially impact the effects of *C. spectabilis* on nematodes. However, the effects of AMF on roots exudates of *C. spectabilis* are totally unknown.

In **chapter 3**, we therefore compared the effects of root exudates of AMF-colonized and non-colonized *C. spectabilis* plantlets on the chemotaxis and viability of *R. similis*. Exudates of non-colonized banana plants were also considered as positive controls. Plantlets were grown *in vitro* to avoid any confounding effects with potential microbial contaminants and their exudates were produced under hydroponic *in vitro* culture conditions. Nematodes chemotaxis was tested in presence of different combinations of root exudates and their viability in presence of different concentrations of roots exudates.

Sixty nine % of the nematodes were attracted by the banana roots exudates when compared to deionized sterilized water, while in presence of roots exudates of AMF-colonized or non-colonized *C. spectabilis* plantlets they moved preferably in the direction of *C. spectabilis* exudates. Interestingly, the nematodes slightly preferred to move in the direction of AMF-colonized *C. spectabilis* roots exudates when confronted to deionized water or banana exudates, even if the differences with the exudates of non-colonized *C. spectabilis* were not significant. Nematodes paralysis was observed after plunging them in the roots exudates of *C. spectabilis* (in presence or absence of AMF), while they remained mobile after plunging in banana roots exudates or deionized water. The number of immobile nematodes was highly influenced by the concentration of the roots exudates with no significant difference was noticed between root exudates of AMF-colonized or non-colonized *C. spectabilis* plantlets. Interestingly, after cleaning the nematodes from the exudates, they recovered their mobility.

Previous studies reported that *C. spectabilis* is a poor-host to *R. similis* and that monocrotaline (a secondary metabolite of *C. spectabilis*) is repellent to the migratory nematodes (Jasy and Koshy, 1994; Wang *et al.,* 2002; Thoden *et al.,* 2009; Wang and McSorley, 2012). In our study, the results were slightly different. Indeed, the nematodes moved preferentially in the direction of *C. spectabilis* exudates (in presence as well as absence of AMF) when confronted to banana exudates. In **chapter 2**, *C. spectabilis* was used as push pull plant in association or not with AMF. In this experiment, a few *R. similis* individuals were observed in the roots of *C. spectabilis* supporting its poor host ability and the nematodes were not able to multiply. Interestingly, *C. spectabilis* was also reported to be a trap crop for *Meloidogyne sp.* and *H. glycines* (Kushida *et al.*, 2003; Viaene *et al.*, 2013), meaning that the nematodes were attracted by *C. spectabilis* but unable to complete their life cycle. The results observed in **chapter 3** (i.e. root exudates of *C. spectabilis* attracting nematodes) may potentially suggest that *C. spectabilis* exudates had a similar trap crop effect on *R. similis* than reported with *Meloidogyne sp.* and *H. glycines.*

Root colonization by AMF did not influence the direction of nematodes. Indeed, when root exudates of colonized *C. spectabilis* plantlets were opposed to root exudates of non-colonized *C. spectabilis*, a similar number of nematodes moved in both directions. Therefore, it is doubtful that AMF (under our cultural conditions) might influence nematode movement by modification of the root exudates of *C. spectabilis*.

Interestingly, nematodes incubated in exudates of *C. spectabilis* loss their ability to move. This was supported by the study of L'Etang (2012) who reported that *R. similis* and *M. arenaria* were paralyzed after 48 h bathing in root exudates of *C. spectabilis*. Osei *et al.* (2010) noticed that monocrotaline, a potentially nematotoxic compound identified in *C. spectabilis*, inhibited the movement of juveniles of *Meloidogyne sp*. These results suggested a probable presence of monocrotaline and/or other inhibitory molecules in the exudates of the roots of *C. spectabilis* in our experiment. However, the biochemical profile of roots exudates was not investigated in our thesis. Curiously, the nematodes recovered their vitality after cleaning from the exudates suggesting that the inhibitory molecules had a nematostatic rather than nematocidal effect. This was also observed by L'Etang (2012) with *R. similis* and *M. arenaria* incubated during 48 h in roots exudates of AMF-colonized *C. spectabilis* plantlets.

VII. CONCLUSION AND PERSPECTIVES

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Conclusions

Banana is a major staple food for rural people in sub-Saharan Africa, Latin America and Asia and belongs to the top five of fruits most consumed worldwide. Its production is threatened by nematodes, which control, in the last decades, relied mainly on nematicides. These chemicals may have numerous negative effects on human health and environment, necessitating the development of alternatives control measures. Among these are the use of clean planting material, paring of the corm, fallow and rotation as well as cropping with non-host companion plants.

Biological control agents such as arbuscular mycorrhizal fungi (AMF) are also receiving an increased attention by scientists and practitioners all over the world. Their role in increasing plant growth and tolerance/resistance to numerous pests (e.g. nematodes) (Pinochet *et al.*, 1996; Perry and Moens, 2013) and diseases (e.g. *Cylindrocadium spathyphilli*, *Mycosphaerella fijiensis*) is widely reported in the literature (Declerck *et al.*, 2002b; Oye Anda *et al.*, 2015). So far, most studies were conducted under pot conditions, while an increasing number are considering *in vitro* cultivation systems such as the one developed by Voets *et al.* (2005) for potato and extended by Koffi *et al.* (2009) to bananas. These highly-controlled systems offers wide perspectives to investigate the effects of AMF on the increasing resistance/tolerance of bananas against phytopathogenic nematodes. Apart from the application of biocontrol microorganisms, the use of push pull plants is also recommended in the control of nematodes. *Crotalaria spectabilis*, for instance, is grown as a cover crop and green manure, is highly mycotrophic and poor host for *R. similis*.

In this thesis, we investigated the role of the AMF *R. irregularis* MUCL 41833 alone or in combination with *C. spectabilis* in decreasing the incidence of *R. similis* in banana. Three experiments were conducted either *in vitro* or in pots with three central questions addressed:

1. Does the AMF *R. irregularis* MUCL 41833 decrease the pressure caused by *R. similis* in banana plantlets cv Yangambi km5?

This question was addressed using the MDP and H-AMP *in vitro* culture systems developed by Voets *et al.* (2005) and Koffi *et al.* (2009). The multiplication rate of the nematodes was drastically decreased within the roots, while no effects of the nematodes were noticed on the AMF in roots or medium. These results demonstrated thus the role of AMF in reducing the incidence of *R. similis* in Yangambi km5 (a partially-resistant cultivar) and the importance of having plantlets pre-colonized by the fungus prior to infestation by the nematode. This study further demonstrated that AMF exert biocontrol effects either on a partially-resistant (Yangambi km5) cultivar (in our study) or highly-susceptible (Grande naine) cultivar (in the study of Koffi *et al.*, 2013a). Several mechanisms may be involved among which competition within and outside the roots and possibly the elicitation of plant defense mechanisms upon colonization by the AMF.

2. Does the combination of a push pull plant (i.e. *Crotalaria spectabilis*) and AMF impact the population of nematodes in banana plantlets?

The split-pot microcosm system developed to grow banana and *C. spectabilis* in the same pot without having they roots mixed demonstrated either the effects of both organisms separately on nematode development and their even more pronounced effect when combined. Indeed, the concomitant use of AMF and *C. spectabilis* impacted all the developmental stages of the nematodes, decreasing their population and multiplication rate in banana roots more than the AMF or *C. spectabilis* alone. Even though this was not significantly different from the AMF and *C. spectabilis* alone, a significant decrease in the RNI was noticed with the combination as compared to the organisms alone demonstrating the potential to consider AMF and *C. spectabilis* together.

3. Are the allelochemical molecules produced by *C. spectabilis* influenced (quantitatively and qualitatively) by AMF colonization?

This hypothesis could neither be excluded nor confirmed. Nematodes were more attracted by exudates of *C. spectabilis* (in presence as well as absence of AMF) when opposed to banana exudates, suggesting the possibility for the presence of attractant molecules. Considering *C. spectabilis* as a trap crop for sedentary nematodes in the literature, the attraction effect of its roots exudates observed in our study suggest its potential role as trap crop for *R. similis*. Curiously, nematodes were paralyzed when bathing in roots exudates of *C. spectabilis* (in presence as well as absence of AMF), while they remained mobile in exudates of banana roots or in water. This effect was concentration-dependent and nematostatic. Indeed, the nematodes recovered their mobility after cleaning from the *C. spectabilis* exudates. The impact of AMF on exudates composition could thus not be ascertained nor excluded and further in-deep analysis of biochemical profile is required.

In conclusion, we have demonstrated that the AMF *R. irregularis* was able to decrease the population of *R. similis* and reduce their impact on roots in the partially-resistant banana cultivar Yangambi km5 as was earlier demonstrated with a highly-susceptible banana cultivar Grande naine. Their combination with the push pull plant *C. spectabilis* increased even more this effect by reducing drastically the surface of necrotic roots in bananas. The mechanisms are probably multifold and root exudates appears central, although, we could not demonstrate that the AMF impacted, nor quantitatively neither qualitatively, these exudates. All our results suggests that the combination of AMF and *C. spectabilis* is an innovative strategy in IPM, although field trials needs to be conducted to confirm this statement.

Perspectives

In this work, we clearly demonstrated the role of the AMF *R. irregularis* and the push pull plant *C. spectabilis* in decreasing the incidence of *R. similis* in banana. We explored the impact of the fungus on exudates of *C. spectabilis* as a potential mechanism explaining the higher biocontrol effect but were unable to demonstrate indubitably such effect. Moreover, it appeared that the exudates of *C. spectabilis* were attractive to the nematode which contradict numerous studies. Therefore, three major questions needs to be addressed in the future:

1. What are the mechanisms involved in the interaction between the AMF and *C. spectabilis* to explain the higher resistance/tolerance of bananas against *R. similis*?

Both the AMF and push pull plant decreased the impact of nematodes on banana roots and their concomitant use increased this effect even more. However, it is unknown if the effect is an addition of the often reported effects of the two organisms separately or if one specific mechanism is boosted by the interaction between the AMF and *C. spectabilis*. In other words, does the AMF increase the push pull effect of *C. spectabilis* or conversely, does the push pull plant increase the efficiency of the AMF in controlling the nematodes. Several assays could be set up *in vitro* to answer these questions. Interestingly, it has been reported that AMF mycelium linking plants below-ground may function as plant-plant underground communication conduits for signaling molecules involved in the control of interactions between plants and other organisms. For instance, induced defense signals are transferred from fungal pathogen-infected or insect-attacked plants to healthy neighboring plants connected by common mycelium networks (CMNs) (Babikova *et al.*, 2013; Song *et al.*, 2014). The nature of these compounds is unknown. It is not excluded that similar signals may be transferred from *C. spectabilis* to the banana plant via the CMN of the AMF. It would be of importance to investigate this by growing *C. spectabilis* with AMFs in the field.

2. Does AMF modify the root exudates composition (and specifically molecules involved in the bioprotection) of *C. spectabilis*?

The role of AMF on the qualitative and quantitative composition of root exudates of *C. spectabilis* remains unknown. However, it is not excluded (as shown for many other plants), that AMF may impact this composition and thus the efficiency of *C. spectabilis* against *R. similis* or other nematodes. Unfortunatley, our results cannot argue for this and further studies are necessary to decipher the biochemical composition of root exudates of plants colonized by the AMF as compared to non AMF-colonized plants. It is thus suggested to investigate this important question, which further complement the question above on the possibility of specific molecules or signals being transported by the AMF from *C. spectabilis* to banana.

3. Does AMF and *C. spectabilis* impacts *R. similis* within the field?

The two questions above are fundamental questions that could be answered under strict controlled conditions. However, in parallel to increasing our basic knowledge on the biocontrol effects of the AMF and *C. spectabilis* on *R. similis*, it is also of prime importance to conduct field trials to compare the effects of both strategies (i.e. AMF or *C. spectabilis*) and their combination on the disease incidence caused by *R. similis* (and other nematodes) in banana. This could be achieved in soils having different population densities of nematodes and under different agricultural practices (in the context of an integrated pest management practice) to decipher under which conditions the push pull plant and/or AMF are the more efficient.

Answering these questions will increase our knowledge on the indubitable positive effects of AMF and *C. spectabilis* in decreasing the incidence of *R. similis* in bananas and on their potential for field application concomitantly to other strategies of ‘bio’-control.

VIII. OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

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Scientific publications

Published

1. Anene, A. and Declerck, S. 2016. Combination of *Crotalaria spectabilis* with *Rhizophagus irregularis* MUCL 41833 decreases the impact of *Radopholus similis* in banana. Applied Soil Ecology, 106: 11-17.
2. Anene, A., Koffi, M.C., Vos, C. and Declerck, S. 2013. Rhizophagus irregularis MUCL 41833 decreases the reproduction ratio of Radopholus similis in the banana cultivar Yangambi km5. Nematology 15, 629–632.

Submitted

3. Anene, A. and Declerck, S. 2017. Root exudates of Crotalaria spectabilis colonized or not by Rhizophagus irregularis impacts the mobility and vitality of the nematode Radopholus similis. Mycorrhiza

Conference participation

- 65th ISCP: International symposium on crop protection. Ghent University. May 2013
- ISHS-Promusa Symposium: agroecological approaches to promote innovative banana production systems. Agropolis International; Montpellier, France. October 2016

Poster:

Anene A. and Declerck S. *Crotalaria spectabilis* combined to *Rhizophagus irregularis* MUCL 41833 decreases the incidence of *Radopholus similis* in banana. ISHS-Promusa Symposium Montpellier, France. October 2016

Teaching and Student supervision

Teaching

1. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi: ***Roots stainng***. Louvain la Neuve, Belgium. May 2014.
2. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi: ***Roots stainng***. Louvain la Neuve, Belgium. May 2015.
3. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi: ***Roots stainng***. Louvain la Neuve, Belgium. May 2016.
4. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi: ***Plants systems***. Louvain la Neuve, Belgium. October 2016.

Supervision

Supervision of one PhD student (Louis Raymond Gbongue) for one year training on: Impact de l'endomycorhization et la silice sur la cercosporiose.

Louis-Raymond Gbongue is a PhD student from Laboratoire de Biotechnologie Végétale et Microbienne/Département Agriculture et Ressources Animales/Ecole Supérieure d'Agronomie (ESA)/UMRI Des Sciences Agronomiques et Génie Rural/Ecole Doctorale Polytechnique de

Yamoussoukro/Institut Nationale Polytechnique Félix Houphouët-Boigny (INP-HB) de Yamoussoukro/Côte d’Ivoire.

IX. REFERENCES

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