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STUDY ON THE INTERACTION BETWEEN ENDOMYCORRHIZAL FUNGI AND *RADOPHOLUS SIMILIS* POPULATIONS

Intermediate report for the accomplishment of the Bio-engineer thesis (KUL).

Presented by

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Abstract

The interaction between the burrowing nematode *Radopholus similis* and the endomycorrhizal fungus *Glomus* sp. on banana was studied in a pot-experiment. Two different populations were used, an aggressive population originating form Ivory Coast (CIV) and a less aggressive population originating from Australia (AUS). No increase in plant growth was observed when mycorrhiza were inoculated. The number of nematodes per gram of roots inoculated with CIV was significantly higher than in roots inoculated with AUS. These results correspond to the aggressivity of the populations. Nematodes did not affect the mycorrhizal colonization.

In a short term pot-experiment, the initial penetration of two *R. similis* populations with a different aggressivity was investigated after 24 hours and 48 hours. Again a population with a high aggressivity (Cameroon, CMR 12) and a less aggressive population were used. Because of a too low number of nematodes that penetrated the roots, no observation could be made.

To have a better understanding in how mycorrhiza can have an influence on the penetration of nematodes in the roots of banana, more controlled conditions were developed. For the third experiment, excised roots placed in a Petri dish with sand were studied. This experimental setup made it possible to have a closer view on the initial penetration of the nematodes in the roots. Different treatments were performed and different experimental parameters were checked.

This experimental setup seemed to have some shortcomings so it needs further development.

Introduction

In most regions of the world, nematodes are recognized as important pests of bananas. Banana nematodes attack the root and corm tissues causing a reduction in mechanical and physiological function of the root system. Annual yield losses caused by nematodes on banana are estimated at about 20 % of the production worldwide.

Nematode management is usually based on chemical control. Nevertheless the negative sides of chemical nematicides as their price and their high environmental toxicity makes it interesting to search for other possibilities like control by biological antagonists.

During the last decades, many studies have been carried out on the use of vesicular-arbuscular mycorrhiza as a possible biological control agent. A review of the literature indicated that the majority of host/fungus/nematode interactions resulted in findings of increased host plant tolerance to nematode damage in mycorrhizal plants as compared with non-mycorrhizal plants. Every host/fungus/nematode interaction however seems to be unique, making this subject one of serious complexity. Every mycorrhizal strain in combination with his host and a nematode population has to be investigated separately.

The experiments performed in this work are part of this complex unity.

In the first experiments two different mycorrhizal strains and three different nematode populations were used to investigate the interaction between mycorrhiza and nematodes. These experiments were performed in semi-controlled conditions (climatic chamber).

In a further experiment a new experimental setup was developed in more controlled conditions (Petri dish). The aim of this experiment was to have a first approach on the mechanisms involved in the penetration of nematodes in banana roots. In this small scale experiment the exact place of inoculating the nematodes could be controlled and thereafter the exact site of penetration could be observed *in situ*.

Chapter 1: Overview of the literature

1.1 Banana

Taxonomy and classification

The banana plant (*Musa* spp.) is a perennial monocotyledonous that belongs to the family of the *Musaceae*. This family is a member of the order Scitamina (Figure 1.1) and is composed of only two genera: *Musa* and *Ensete* (Simmonds, 1966). The genus *Musa* contains 50 species classified into 4 sections: *Rhodochlamys, Callimusa, Eumusa* and *Australimusa* (Cheesman, 1948; Simmonds & Shepherd, 1955), but only the latter two are of any economic importance. The section of *Eumusa* is the largest and the most geographically widespread of the genus and contains the majority of the edible bananas. The inedible seed bearing diploid species (2n=22) *Musa acuminata* Colla (AA) and *Musa balbisiana* Colla (BB) are the parents of the agricultural produced banana (Gowen, 1988) of this section. Over time mutation led to the evolution of parthenocarpy (i.e. the ability to form fruit without fertilization) and sterility, resulting in the production of seedless and edible fruit. Furthermore the absence of meiosis in the female gametes led to the formation of triploids and tetraploids (Stover & Simmonds, 1987). So bananas can be diploid, triploid or tetraploid. Most of the cultivated varieties however are triploid (3n=33) including the commercial Cavendish AAA varieties (Annex 1).

Botany

The banana consists of a branched, underground stem (the corm) with roots and vegetative buds, and an erect pseudostem composed of tightly packed leaf bases (Figure 1.2).

Banana plants may have as many as 300 - 500 primary roots. They are about 5 - 8 mm in diameter and can reach a length of about 10 meters. They usually develop in groups of three or four from the corm. The roots are white in color when young and healthy, and become corky with age. From the primary roots, a system of secondary and tertiary roots and root hairs develops.

The vegetative buds form shoots that can form new stems, called suckers. 5 to 20 suckers can arise from each stem each year but only one sucker is usually selected by the farmer

to grow and regenerate the plant. The other suckers are either removed physically or their apical meristems destroyed chemically. This is necessary because they compete with the mother plant in nutrients resulting in reduction of bunch size.
In commercial plantations, densities of up to 2000 plants per hectare are maintained by regular pruning of suckers, leaving one to replace the parent plant which is cut down after harvest (Gowen, 1988).

The apical meristem is located in the center of the pseudostem close to the ground level. It gives rise to a succession of leaf primordia which grow upwards and give rise to the new leaves. The leaves emerge from the middle of the pseudostem as rolled cylinders which gradually unfold (Figure 1.3). Leaves emerge at different rates depending on the cultivar and environmental conditions. In summer time in tropical regions, the rate of leaf emergence can go up to 3,5 - 3,8 leaves $month^{-1}$ and in winter in the cool subtropics, it can fall to 0,1 - 1,2 leaves $month^{-1}$ (Jones, 2000).

The banana tree is monocarp, what means that it flowers only one time in its existence. At a certain stage of plant development, about 7 months after planting, the apical growing point stops initiating leaves and develops an inflorescence. The flower stalk or peduncle elongates and the inflorescence emerges. Flowers are arranged in nodal clusters; the basal nodes bearing female flowers and the upper nodes male flowers (Figure 1.2), which are tightly enclosed in bracts. Only the female flowers possess a pistil that is large enough to become a fruit.
Flowering to maturity takes approximately 16 weeks, producing edible fruits. The shape, size and taste vary between cultivars. Each fruit is referred to as a "finger" and the bunches they form are "hands". A good bunch consists of at least eight hands of fifteen fingers (Samson, 1986) and may weigh up to 30 kg (Gowen, 1988) or sometimes even more.

Climate and soils

Bananas are cultivated over a variety of soil types and climates. The most extensive areas of commercial banana production fall within 30° latitude N and S, where an optimum in temperature can be reached and the temperature fluctuations from day to night and from summer to winter are comparatively small (Shanmugavelu *et al.*, 1992). For production

they require a minimal monthly rainfall of 100 mm and a mean minimum temperature above 15 °C. At 12 °C the production is inhibited and the banana will die.

There are a lot of soils suitable for growing bananas but for best yields bananas should be grown on deep (> 60 cm) and freely draining soils. Compact clay soils should be avoided (Stover & Simmonds, 1987). Annex 2 gives an overview of the diversity of soils that can be suitable and their main associated constrains apart from topography and climate (Delvaux, 1995).

Nutrition

As bananas are high demanding crops, the high amounts of nutrients for maximum growth generally cannot be supplied by the natural reserves of the soil. So to maintain high yields, soil fertility has to be improved by adding organic manure and/or mineral fertilizers.
Large amounts of N and K are applied either by hand or in the irrigation water. The rate of application depends on climate, soil type, variety, management practices and yield.
High-input cropping systems however result in a rapid environmental degradation so soil fertility research is clearly needed to meet the demand for sustainable agricultural production (Scholes *et al.*, 1994).

Economic importance

The banana is cultivated in more than 120 countries, especially in the Latin American-Caribbean region, Africa and the Asian-Pacific region (Jones, 2000) (Figure 1.4). After rice, wheat and maize, the production of bananas is the most important in the world (CGIAR, 1992, 1993). The annual production is estimated at approximately 88 million tons, but only about 15 % reach the world export market. The remaining 80 million tons are consumed locally, highlighting the importance of this food crop in many communities (Figure 1.5; Lescot, 1999). Bananas are a major staple food crop for 400 million people throughout the humid and the subhumid tropics and for another 600 million people they give 25 % of the basic needs.

The cultivation of bananas can be divided into two types of production:

- The industrial production of bananas (dessert bananas), which represents 41 % of the world production (Lescot, 1999). The cultivation of these bananas is often oriented towards very large plantations, long plantation life, extensive technical infrastructure, use of high amounts of pesticides and high quality fruit (Robinson, 1995). Their exploitation relies on monoculture, often without rotation.
- The production of bananas for self-consumption or for sale in local markets (59 % of the world production). This extensive production exists mainly on cooking bananas, and for only a small percentage on dessert bananas. The cultivation takes place with the local farmers, on small parcels.

Banana is an attractive perennial crop for farmers in developing countries. The banana plant can grow in a wide range of environments and the fruit can be produced all year round so it provides a steady cash income or supply of nutritious food.

Banana pests and diseases

Diseases are among the most significant constraints to banana production in the subtropics. The lack of genetic diversity in commercial banana production and the intense monoculture of this crop renders it vulnerable to diseases.

In the subtropics, Fusarium wilt (Panama disease), caused by *Fusarium oxysporum* f. sp. *cubense*, is a widespread and important disease (Ploetz, 1998). This soil borne pathogen penetrates the root and slowly spreads to the corm, it blocks xylem vessels resulting in the yellowing and collapse of outer leaves. Effective fungicides do not exist for this fatal disease but cultivars from the Cavendish AAA subgroup were found to have resistance to this pathogen.

Other important diseases of the banana plant are usually not fatal, but can limit production severely. Bunchy top disease, caused by the banana bunchy top virus is by far the most serious viral disease and can have a devastating effect on crops. Fortunately it has a somewhat restricted geographic distribution so it can be easily controlled.

Some important leaf diseases are Sigatoka and freckle, caused by the fungi *Mycosphaerella musicola* and *Guignardia musae* respectively. These and other foliar diseases can cause serious damage during periods of high rainfall but can be controlled with protectant and systemic fungicides and plantation sanitation.

Most bacterial diseases of bananas can be grouped into two categories: vascular infections caused by *Pseudomonas solanacearum* and related organisms, and diseases caused by soft-rotting *Erwinia* species (Gowen, 1995). Both groups include diseases with similar symptoms, but which vary in importance for different regions or different hosts. An important bacterial disease of banana is Moko disease, caused by strains of *P. solanacearum*. Like fusarium wilt, this disease causes a vascular wilt and discolors the vascular system. It even causes a discoloration of the fruit.

Insect pest can either damage the corm (for example the banana weevil *Cosmopolites sordidus*), the fruits (for example the banana moth *Opogona sacchari*) or the leaves (for example species of Lepidoptera). One of the most important pests on banana are the plant-parasitic nematodes. They damage the root system resulting in depletion in uptake of nutrients and water. They are widespread and cause severe crop losses in commercial banana plantations. Four nematode species are the most important on bananas: the burrowing nematode, *Radopholus similis*, the lesion nematodes, *Pratylenchus coffeae* and *P. goodeyi*, and the spiral nematode, *Helicotylenchus multicinctus* (Gowen & Quénéhervé, 1990). The burrowing and lesion nematodes are the most damaging pests in the tropics, but the spiral nematode is more important in the subtropics.

1.2 Nematodes

1.2.1 Nematodes attacking several crops

Introduction

Nematodes constitute one of the most important and abundant group within the animal kingdom (Table 1.1). They are widespread and they can occupy more different niches and habitats than any other organism. Their small size, resistant cuticle and ability to adapt to severe and changing environment give them the capacity of surviving in a wide range of environmental conditions. They live in lakes, rivers and oceans all over the world and function in almost all the types of soils, feeding on microorganisms and microscopic plants and animals. Numerous species of nematodes attack and parasitize animals (even humans) in which they cause various diseases (Wallace, 1963).

Plant-parasitic nematodes are the most damaging pests of all banana varieties and are one of the greatest threat to crops throughout the world. Nematodes alone or in combination with other soil microorganisms have been found to attack almost every part of the plant, including roots, corm, stems, leaves, fruits and seeds. The damage they can cause is estimated at eighty billions US dollars in the world each year (Barker *et al.*, 1994), and it is considered that all crops are damaged by at least one species of nematode.

Morphology and anatomy

Besides their wide ecological diversity, the different species of nematodes are fairly alike in structure. Plant-parasitic nematodes are small, 300 to 1000 μm long (with some up to 4 mm), by 15 - 35 μm wide, what makes them essentially invisible to the unaided eye. They are, in general, eel-shaped and round in cross section, with smooth, unsegmented bodies, without any appendages.

Nematodes consist of an elongate stomach and reproduction system inside a resistant multi-layered outer cuticle (usually transparent). For feeding, nematodes use a stylet: a hard, sharp spear. Figure 1.6 shows the general anatomy of a plant-parasitic nematode.

Life cycle and reproduction

Plant-parasitic nematodes have a fairly simple life cycle which has six stages: the egg, four juvenile stages, and adult. Male and female nematodes occur in most species, and both may be required for reproduction. Parthenogenesis (i.e. reproduction without males) however is common, and some species are hermaphroditic.

The length of the life cycle varies considerably with each species, its host plant, and ecological parameters (climate, altitude). Rates of activity, growth, and reproduction increase as soil temperature rises from about 10 °C to about 32 °C. Minimum generation time is about 4 weeks for many nematodes under optimum conditions (about 27°C for many nematodes in tropical regions).

The number of eggs deposited by a female varies among species and is affected by the habitat. Most species produce between 50 and 500 eggs, but a few species sometimes produce several thousand eggs per female. Eggs of some species can survive without hatching for years but hatch quickly when a host plant grows near them.

Types of nematodes

In general, based on their biology, four types of plant-parasitic root nematodes can be distinguished (Speijer & De Waele, 1997):

Ectoparasitic nematodes are nearly all migratory i.e. they are mobile and can freely leave the roots towards the soil. They live completely outside the host plant and feed superficially at or very near the root tip or on root hairs. Some however have a stylet long enough to reach several cells deep. Examples are Sting nematode (*Belonolaimus* spp.), Stubby-root nematode (*Trichodorus* spp.) and Ring nematode (*Criconemella* spp.).

Sedentary semi-endoparasitic nematodes have juveniles and young females that migrate and feed ectoparasitically, saccate females whose front end of the body is buried in the root tissue while the rear end remains outside and eggs that are laid into a gelatinous matrix secreted by the female (Stirling, 1991, Agrios, 1997). *Rotylenchulus* is recognized as a common parasite of secondary and tertiary roots, but not the primary roots of Cavendish varieties (Edmunds, 1970). Examples are Reniform nematode (*Rotylenchulus* spp.) and Citrus nematode (*Tylenchulus* spp.).

Migratory endoparasitic nematodes live completely inside the host plant but are able to move freely between the roots and the soil. They generally live and feed in tender tissues such as the root cortex. They burrow through the tissue, breaking open many cells after they have finished feeding on them. Examples are Spiral nematode (*Helicotylenchus* spp.) in banana, Lesion nematode (*Pratylenchus* spp.) and Burrowing nematode (*Radopholus* spp.).

Sedentary endoparasitic nematodes also live inside the host plant but the females become sedentary. These nematodes have a complex interaction with the host plant because they induce normal plant cells to form specialized feeding structures. Mature females generally produce large numbers of eggs which are retained in the body or accumulated in masses attached to their bodies.
Externally, the effect of root-knot nematode parasitism is visible as swelling of the root which is commonly called gall or knot. Examples are Root-knot nematode (*Meloidodyne* spp.) and Cyst nematode (*Heterodera* spp.).

Interrelationships between nematodes and other plant pathogens

Many nematode species live in the soil, along with fungi and bacteria, which also can be pathogenic to plants. In several cases an association develops between nematodes and certain of these other pathogens or parasites. This can result in a pathogenic potential that sometimes appears to be far greater than the sum of the damages either the pathogens can produce individually (Agrios, 1997).

Nematode species that cause serious damage to banana

Many nematode species are present in roots of banana. They all belong to almost 150 species and more than 40 genera (Gowen & Quénéhervé, 1990). Only a few however cause damage that is of economic importance:

Radopholus similis (the burrowing nematode) occurs widely in tropical and subtropical regions of the world. In most banana-growing areas it is the most important banana root pathogen, especially in intensive plantations. It causes the banana black rot, blackhead toppling disease, or decline of banana (see paragraph 1.4).

Pratylenchus coffeae and ***P. goodeyi*** (the lesion nematodes) can attack the roots of all kinds of plants (Gowen, 1993). *P. coffeae* is the most widespread species on cooking bananas in Central America and the Caribbean Islands. *P. goodeyi* is considered to be the most widespread and most damaging nematode on bananas in the African highlands and the Canary Islands (Sarah, 1989). Lesion nematodes reduce or inhibit root development by forming local lesions on young roots, which may then rot because of secondary fungi and bacteria.

Helicotylenchus multicinctus (the spiral nematode) is found in all varieties of bananas throughout the world and commonly occurs in mixed populations with other nematodes (Gowen & Quénéhervé, 1990). In some subtropical regions where *R. similis* is absent, this species may be the major nematode concern. The damage symptoms on banana caused by *H. multicinctus* are more superficial than the damage observed with other serious root parasites such as *R. similis*.

Meloidogyne **spp.** (the root-knot nematodes) have a worldwide distribution and attack many economically important crops (Gowen & Quénéhervé, 1990). The species most commonly found associated with bananas are *Meloidogyne incognita*, *M. arenaria*, *M. javanica* and *M. hapla*. The most obvious symptoms of the root-knot nematode are galls and swellings on primary and secondary roots (De Waele, 2000). However, because they do not cause plants to topple, these nematodes are not thought to be as important as the more destructive lesion forming nematodes, which in mixed populations are generally dominant (Gowen, 1995).

1.2.2 *Radopholus similis*

Taxonomy and distribution

Radopholus similis (Cobb, 1893) Thorne 1949, belongs to the Class Nematoda, Family Pratylenchidae, Subfamily Radopholinae, Genus Radopholus. It was first described by Cobb in the Fiji Islands in 1893 where it had been found to cause necrosis of banana roots since 1890. Its importance as a pest of commercial bananas however did not receive attention until 60 years later when descriptions of the symptoms on Cavendish bananas were published (Loos & Loos, 1960; Stover, 1972).

Radopholus similis is present in almost all banana producing regions of the world (West and Central Africa, Central and South America, Hawaii, Florida, Australia) (Figure 1.7). The burrowing nematode however seems to be absent from isolated zones where cultivation occurs such as the Cape Verde Islands and the Jordan Valley in Israel. It is also absent from the highest-altitude zones where banana is cultivated, such as the highlands of central and eastern Africa, Southern Mexico and Mozambique, and from the highest latitude zones, such as the Canary Islands, Cyprus, Taiwan and South Africa (Gowen & Quénéhervé, 1990; Sarah, 1989; Stover & Simmonds, 1997; Sarah, 2000).

During the late 1950's to early 1970's 'Gros Michel' banana plantations were replaced by the wilt resistant Cavendish cultivars, which were more susceptible to the burrowing nematode. As a result, *Radopholus similis* became the most damaging plant-parasitic nematode of bananas (Román, 1986). Its widespread distribution is believed to be due to the transference of infected plant material from country to country (Orton & Siddiqi, 1973).

Biology and life cycle

Radopholus similis is a vermiform migratory endoparasitic species which is able to complete its life cycle within the root cortex.

The life cycle consists of an egg stage, four juvenile stages and the adult stage. At 24 -32 °C the life cycle takes 20 - 25 days; fertilization is usual but also parthenogenesis may occur (Orton & Siddiqi, 1973). Only the females (Plate 1) and juveniles penetrate and parasitize the host tissue; males (Plate 2) are probably non-parasitic. Penetration occurs mostly near the root tips, but they can invade along the entire length of the root. After entering the roots of banana, the nematodes occupy an intercellular position in the

cortical parenchyma, 1 - 4 cells beneath the epidermis. There they feed on cytoplasm of nearby cells until only the nucleus and a small amount of cytoplasma remain. Then the nucleus disintegrates, the primary cell wall ruptures and a cavity forms into which the nematode moves. Necrosis can extend to the entire corm cortex (black head disease), and roots, but the root stele is usually not damaged except occasionally when invasion occurs in young plants (Mateille *et al.*, 1994; Valette *et al.*, 1997). It is in the infected tissue that females lay their eggs. Females produce an average of 2 eggs a day (range 0,5 - 6 eggs/female/day). Eggs hatch occurs in 8 to 10 days, and the four juvenile stages occur over the following 10 to 13 days.

Symptoms and Pathogenicity

As the burrowing nematode feed in the cortex, it results in lesions and cavities which destroy root and corm tissues. This necrosis appears as a reddish-brown discoloration. The lesions can vary from small spots to large areas of necrotic tissue. These cavities in the roots and corm reduce water and mineral uptake which results in a reduction of plant growth and development and may lead to severe reduction of the bunch weight and an increase of the time period between two successive harvests (Gowen, 1975). Furthermore, this destruction results in a weakened root system so trees can fall (toppling disease) because of their own weight or strong windstorms.
Crop losses depend on several factors, including the pathogenicity of the local burrowing nematode populations, associated pathogens (including other nematode species), banana cultivar, climatic conditions and soil factors (especially fertility) (Sarah, 2000).

Pathogenicity: The results of many studies confirm the existence of large biological variability and difference in pathogenicity among geographically separated populations of *R. similis*. These differences in pathogenicity seem to be clearly linked to reproductive fitness (agressivity) in plant tissues. Isolates from Uganda, Ivory Coast, Costa Rica, Cameroon and Guinea have been shown to have higher multiplication rates than those from Martinique, Guadeloupe, Sri Lanka and Australia (Sarah *et al.*, 1993; Fallas & Sarah, 1995; Fallas *et al.*, 1995; Fallas *et al.*, 1996; Hahn *et al.*, 1996). Worldwide assessment of this variability would be highly useful to banana breeding programs for incorporating resistance to nematodes.

Associated pathogens: Necrosis of root and corm tissues is accelerated if secondary parasites and pathogens, such as fungi and bacteria are present. Fungi commonly associated with burrowing nematode lesions are *Cylindrocarpon musae*, *Acremonium stromaticum*, *Fusarium* spp. and *Rhizoctonia solani* (Laville, 1964; Pinochet & Stover, 1980).

Banana cultivar: Screening studies have shown that some banana cultivars are more susceptible to *R. similis* than others. The cultivar 'Poyo' of the Cavendish subgroup is quite susceptible, the cultivars 'Gros Michel' and its dwarf mutant 'Cocao' are believed to be less susceptible (Wehunt *et al.*, 1978) while 'Yangambi Km 5' (AAA, syn. 'Ibota Bota') has a very strong resistance to *R. similis* (Sarah *et al.*, 1992; Price, 1994; Stoffelen, 2000).

Climatic conditions: Rainfall appears to be a main factor that modulates nematode populations (Jones & Milne, 1982). Too little and too much water lower nematode densities in the roots. Also temperature can be a limiting factor making *R. similis* generally absent in cooler banana-growing areas.

Soil fertility: Studies have revealed that crop losses because of nematode infestations are high in poor and eroded soils, but are much lower when the soil has a high fertility (Sarah, 1989; Sarah, 1995).

Damage and economic importance

Nematode damage is often a limiting factor in banana production, making *R. similis* a pest of substantial economic importance (Sarah, 1989). Yield reduction of 30 - 50 % has been observed in banana growing areas in Coast Rica and Panama. In other banana producing countries yield losses due to nematode attack may range from 10 to 30 %. All over the world, banana companies spend millions of US dollars on nematicides and their application. Furthermore strong winds and heavy rains lead to yield losses due to uprooting or toppling of plants ranging from 104 to 156 bunches ha^{-1} $year^{-1}$ so causing another loss of around 4 million US dollars (Davide, 1994).

1.2.3 Nematode Control

Several methods of effectively controlling nematodes are available, although certain factors, such as costs and types of crops, may influence the types of control methods employed. Control is usually attempted through control with chemicals, such as various types of nematicides. Other control methods are through cultural practices such as use of crop rotation, fallow, and cover crops, by means of physical agents such as heat and flooding or by use of resistant varieties. Finally biological control with the use of antagonistic bacteria and fungi is possible. In practice, a combination of several methods is usually employed for controlling nematode diseases of plants.

Chemical control

Control of nematodes by repeated application of nematicides is an effective method, but can only be used where the economic value of the crop and the environmental conditions are satisfactory. Nematicides are widely used in large plantations for international export market. For local market the use of these pesticides is not possible nor justified.

The nematicides used are generally non-volatile organophosphates or carbamates. They are applied as granules on the soil surface. Emulsifiable compounds are applied as liquid sprays or through irrigation systems (Gowen & Quénéhervé, 1990). Nematicide efficiency, environmental conditions, population dynamics and the pathogenicity or aggressivity of the local strains determine the optimum application time, dose and frequency of applications.

In some banana-growing countries, nematicides are applied on a regular basis and no attempt is made to determine if the treatments are necessary or not. As well as being costly, nematicides are also very toxic and can endanger the environment and human health if used indiscriminately.

Cultural practices

In the tropics and subtropics crops are often grown continuously on the same land. These methods of intensive monocultivation lead to an increase in all pests present in the soil. Crop rotation with non-host plants can be a solution to control *R. similis* because it can only survive in soil for 9 to 18 months in the absence of a host plant (Loos, 1961). Crop rotations with sugarcane have met with some success (Loos, 1961). Milne & Keetch

(1976) have published a list of 44 non-host plants of *R. similis* in South Africa, which thus can be used in crop rotation with banana.

Soil fertility is an important factor for improving plant tolerance to nematode damage (Sarah, 1995). Soil ploughing allows the root system to develop better before planting and incorporation of organic matter and fertilizers into the soil improve plant growth. It even seems that organic matter has a direct action against nematodes, both because of its chemical composition and because of the species of microorganisms that develop when organic matter is applied to the soil (Rodriguez-Kabana *et al.*, 1987).

The development of micropropagation techniques is recently under study. Indeed, micropropagated plants are free of nematodes, and if planted in a field free of nematodes, plantations can be maintained for a long time without any treatments with high yields. Mateille (1992) reported that fallow and flooded plots replanted with *in vitro* plants contained no *R. similis* within the following 2 years.

Physical control

Severe flooding over a 5 - 7 week period in the Nieky Valley in the Ivory Coast in 1976 and 1982 led to a dramatic reduction in nematode numbers, due to lack of oxygen and the possible generation of toxic sulphur compounds by anaerobic bacteria (Sarah, 1989). Unfortunately in most tropical regions the lack of access to sufficient water and unsuitable topography prevents use of this method.

Another method for eliminating nematodes from the planting material involve a combination of peeling (paring) and a hot-water treatment of the corms. However, hot-water treatments are labor-intensive and require careful monitoring since temperature and immersion times are critical.

Solarization uses the sun's energy to heat the soil through a plastic film (Katan, 1981). Where the soil temperature reaches 47 °C for two hours a day over 6 days, no nematode can survive. However, this technique only treats the soil to a few centimeters deep and can only be used in regions where, or during seasons when it is not generally overcast. Because of these restrictions this technique is of limited interest in the humid tropics.

Control by resistant varieties

Growing nematode resistant or tolerant varieties is considered as an effective and sustainable method for nematode management. Fogain & Gowen (1997) demonstrated in field trials that population levels of *R. similis* were higher on the root systems of nematicide-treated susceptible cultivars than on an untreated resistant cultivar. Their work shows that genetic resistance can effectively control *R. similis*.

Resistance and susceptibility are relative qualities of a host plant based on comparison between varieties. A host plant may either suppress (resistance) or allow (susceptibility) nematode development and reproduction, or it may suffer little injury (tolerance), even when heavily infected with nematodes (Bos & Parlevliet, 1995).

To find the sources of resistance, research programs are set up to screen the *Musa* gene pool. Up to now screening of banana varieties for nematode resistance is conducted either under field (Wehunt *et al.*, 1978; Price, 1994) or greenhouse conditions (Sarah *et al.*, 1992). Already some accessions are found to be resistant to *R. similis*: the accession 'Yangambi km 5' and 2 'Pisang Jari Buaya'accessions of the *Eumusa* bananas, and the 'Rimina' and 'Menei' accessions of the *Australimusa* group (Stoffelen, 2000).

A better knowledge of mechanisms involved in the resistance of *Musa* to nematodes, leading to the identification of the dominant genes involved, would be undoubtedly helpful for the breeding programs. Recent advances in recombinant DNA and tissue-culture technology have made the transfer of genes from microorganisms, plant or animals to higher plants possible, so engineered nematode tolerant or resistant cultivars are now within reach.

Biological control

Plant-parasitic nematodes have many natural enemies and a number have been considered as possible biological control agents. But despite considerable research effort, no organism is yet routinely used for the biological control of a nematode pest on any crop. Fungal antagonists of nematodes are comprised of a great variety of organisms, which include the nematode-trapping or predacious fungi, endoparasitic fungi, parasites of nematode eggs, parasites of nematode cysts and fungi that produce enzymes and metabolites which are toxic to nematodes (Mankau, 1980; Sikora, 1992).

The nematophagous fungi *Paecilomyces lilacinus* has been found to reduce *R. similis* populations; it is a parasite of burrowing-nematode eggs, juveniles and adults (Davide, 1994). The bacteria *Pseudomonas fluorescens* and *P. putida* have been demonstrated to inhibit the invasion of roots of 'Grand Nain' (AAA) (Aalten *et al.*, 1998).

Recent attempts have been successful in reducing the nematode populations in roots by inoculating the roots with mycorrhizal fungi. Several studies under glasshouse conditions have been undertaken with different mycorrhizal strains and different nematode populations. Although little implementable results are obtained until now, this practice can be promising in the future.

1.3 Mycorrhizal fungi

Introduction

Only in the last decades botanists and mycologists have realized that most terrestrial plants live in symbiosis with soil fungi. The term mycorrhiza, first coined by the German plant pathologist A. B. Frank, literally means "fungus root". It therefore basically designates the symbiotic association between fungi and plant roots.
Mycorrhizal fungi are divided into two major groups, the endo and the ectomycorrhiza. These prefixes refer to whether actual penetration of root cortical cells by the fungal symbiont occurs. Ectomycorrhizal fungal hyphae interweave to form a compact sheath around the root, known as the fungal mantle. Hyphae can penetrate through the epidermis into the intercellular spaces of the cortical cells, replacing the middle lamella and forming an interconnecting network known as the 'Hartig' net. Endomycorrhizal hyphae however grow both inter and intracellularly in the root cortex, after penetration through the epidermis or root hairs. A fungal mantle is not formed although a few hyphae grow along the root surface and branches from these hyphae extend well out into the soil (Rhodes & Gerdemann, 1975).

By far the most common mycorrhizal association is the vesicular-arbuscular type, which produces fungal structures (vesicles and arbuscules) in the cortex region of the root, making them belong to the endomycorrhizal group. VAM-fungi are associated with a greater variety of plant species and are more widely spread geographically and ecologically than other types of mycorrhzial fungi. They live in association with approximately 85 % of herbaceous plants, making mycorrhizal symbiosis a rule rather than an exception.

The principle of symbiosis

A symbiosis refers to an association of living organisms that benefits both partners, enabling them to survive, grow and reproduce more effectively. VAM-fungi simultaneously colonize the roots and their rhizosphere and spread out over centimeters in the form of ramified filaments. This filamentous network dispersed inside as well as outside the roots allows the plant to have access to a greater quantity of water and soil minerals required for its nutrition. In return, the fungus receives from the plant

metabolized nutrients that it is unable to synthesize itself, such as sugars, amino acids and secondary metabolites (Harley & Smith, 1983). The colonized plant is better nourished and better adapted to its environment. It obtains increased protection against environmental stresses, including drought, cold, salinity and pollution (Sylvia & Williams, 1992). In addition, symbiosis tends to reduce the incidence of root diseases and minimizes the harmful effect of certain pathogenic agents (Dehne, 1982).

Taxonomy and morphology

The VAM-fungi belong to the Zygomycetes, and have been recently regrouped in a single order, the Glomales, which include all species capable of living in symbiosis with plants. The VAM-fungi consist of approximately 160 species belonging to 3 families and 6 genera.

VAM-fungi develop a major network of microscopic filaments in the soil. When filaments of these fungus organisms come in contact with a young root, they thread their way between the cortical cells and quickly propagate, forming intracellular arbuscules and, in some cases vesicles (Plate 3). In uniform development, arbuscule formation precedes vesicle formation. Arbuscules, developed from repeated dichotomous branching of intracellular hyphae, are considered to be the major site for nutrient exchange, although this is recently under discussion (Smith & Read, 1997). Vesicles (Plate 4), spherical swellings at the tips of intercellular or intracellular hyphae, probably serve a storage function. Spores (Plate 4) are also differentiated in the soil and in the roots. They act as reserve and propagation organs and are a reference structure for species identification. Figure 1.8 and gives an overview of the different mycorrhizal structures inside and outside the root.

Importance in agriculture

In commercial production systems such as monoculture, most attention is paid to yields by high fertilizer and pesticide inputs and less attention is paid to preserving natural soil resources by establishing or maintaining more sustainable cropping systems. In these environments, naturally occuring VAM-fungi or the introduction of selected species may benefit plants without increasing input costs too much. Including VAM-fungi in integrated crop management could be considered, particularly in situations where soils

are low in available P, fragile or subject to erosion; conditions that are frequent in banana cropping systems (Delvaux *et al.*, 1998).

VAM-fungi are renowned for their growth promoting properties thought to be due to the increased nutrient levels found in mycorrhizal plants compared with non-mycorrhizal plants. The large hyphal network around the roots enlarges the absorbing surface so mycorrhizated plants can take up more of nutrients from the soil then non-mycorrhizated plants (Sanders & Tinker, 1971). They also augment the plant's access to soluble phosphates in the soil because they are especially effective at absorbing phosphorus, which is then transferred to the host (Jaizme-Vega, 1998).

The VAM-fungi affect not only plant development and nutrition, but can also increase the plant's natural resistance to biotic (pathogens) or abiotic (hydric stress, salinity, pollution) imbalances (Hussey & Roncadori, 1982; Sylvia, 1989; Charest *et al.*, 1993). Also in micropropagation practices, VAM-fungi are of important interest. The pre-inoculation of micropropagated plantlets free of pathogens appears to be an appropriate management practice to introduce VAM-fungi in the field (Delvaux *et al.*, 1998). Potential advantages are numerous. First, VAM-fungi are already established in the roots and, will consequently have a competitive advantage over soilborne pathogens (Jaizme-Vega *et al.*, 1997). Second, higher plant growth rates may result in a shorter nursery phase. Third, far less VAM propagules are required to inoculate young plantlets (having fewer roots) than older plants and may result in reduced costs.

The mutualistic symbiosis between the VAM-fungi and banana is non-host specific (Gerdemann, 1968). A single plant species can associate with most (if not all) known VAM-fungi, while a single fungal isolate can infect a vast array of host species (Smith *et al.*, 1992). Experiments however have showed that the efficiency of the symbiosis can differ between different fungus/host pairs (Declerck *et al.*, 1995). Selection of efficient VAM fungus/banana cultivar couples for a given set of field conditions should thus be a consideration in the improvement of sustainability of banana cropping systems.

1.4 Interactions between mycorrhiza and nematodes

Since plant-parasitic nematodes and vesicular arbuscular mycorrhizae are intimately associated in the root system of many plants, it is logical to consider an interaction between these two groups of organisms. The obligatory symbiotic VAM-fungi may stimulate plant growth whereas the obligate plant-parasitic nematodes usually suppress plant growth. The action of VAM-fungi however may not only be one of simply improving growth; they may also play a role in plant protection against certain phytoparasitic agents.

Many interactions between various host plants, VAM-fungi and nematodes have been undertaken but the outcomes of these interactions do not always give results in the same direction. The most common effect found is that VAM-fungi promote resistance or tolerance to nematodes in nematode susceptible plants (Hussey & Roncadori, 1978; Saleh & Sikora, 1984; Smith *et al.*, 1986b; Umesh *et al.*, 1988; Jaizme-Vega *et al.*, 1997; Habte *et al.*, 1999). In some studies however, mycorrhizae seemed to have no effect (O'Bannon & Nemex, 1979; Thompson & Hussey, 1981; Pinochet *et al.*, 1995; Pinochet *et al.*, 1997) or sometimes even more nematodes were observed in the mycorrhizated roots (Kassab & Taha, 1991). Results indicate that the fungal-host plant combination is critical, with each fungal isolate producing different host reactions to a particular plant-parasitic nematode. Also the conditions in which the experiment is performed change between different experiments, giving another explanation for the variability in results.

A number of possible hypotheses are made to explain the beneficial effects of VAM-fungi on plants parasitized by nematodes (Hussey & Roncadori, 1982; Smith, 1987) but the exact mechanisms that are responsible for this resistance or tolerance to nematodes are not well known. Possible hypotheses are:

Increased of root growth and function. VAM-fungi offset the yield loss normally caused by nematodes by expanding the absorptive capacity of the root system thus enhancing the uptake of phosphorus and other nutrients, therefore improving plant vigor and growth (Harley & Smith, 1983).

Alteration of root exudation and root metabolism. VAM-fungi physiologically alter or reduce the root exudates of the host plant. Graham & Menge (1982) cited by Pinior *et al.* (1999) suggested that these alterations induced by mycorrhizal colonization might be linked with the enhanced resistance of mycorrhizal plants towards soilborne pathogens.

The sugar content of a plant is known to have an influence on its disease resistance. Horsfall & Diamond (1975) cited by Suresh & Bagyaraj (1984) reported that disease incidence was greater when host plant sugar levels were low; mycorrhizal plants possess high sugar contents likely to affect their resistance to nematodes.

The amino acid content of mycorrhizal plants is altered; increases in the levels of arginine, phenylalanine, isoleucine, threonine and serine have been recorded (Schenck, 1983; Suresh & Bagyaraj, 1984). Increased levels of two of these compounds, phenylalanine and serine, are thought to reduce the growth and reproduction of the root-knot nematode (Krishnaprasad, 1971 cited by Surech & Bagyaraj, 1984).

Nematode migration might also be impaired through increased lignification and suberization processes (Smith, 1987).

Competition for resourses. Cells colonized by fungal symbionts seemed to be an unfavorable food source for parasitic nematodes. A reasonable explanation for this can be the accumulation of phenolic compounds, hormones and phytoalexines within the tissues of mycorrhizated plants, affecting nematode feeding (Pinochet *et al.*, 1996b).

VAM-fungi are almost totally dependent on soluble carbohydrates produced by the host for their carbon source (Harley & Smith, 1983). Sedentary endoparasitic nematodes are also dependent on their host for their carbon source. Although evidence of host resource competition between VAM-fungi and pathogens is lacking, this hypothesis warrants investigation (Smith, 1988).

Because VAM-fungi and plant-parasitic nematodes occupy similar root tissues, direct competition for space has been postulated and a mechanism of pathogen inhibition by VAM-fungi (Davis & Menge, 1980; Hussey & Roncadori, 1982). However, this hypothesis has not received much attention because many root pathogens infect at the root tip where VAM-fungal structures do not occur (Garrett, 1970; Harley & Smith, 1983). This hypothesis has been further discounted because inhibition of nematode activities on mycorrhizal root systems has occurred with approximately 50 % of the root

system devoid of any mycorrhizal structures (Cooper & Grandison, 1986; Smith *et al.*, 1986a).

These and even more hypotheses about the beneficial effect of mycorrhizae on pathogens have been published, but this subject has to be taken with care and more studies are necessary to draw any conclusion. The only hypothesis that is confirmed until now is that VAM-fungi mainly act through effects on the plant rather than on the nematodes by enhancing the uptake of nutrients thus improving the plant vigor, making it more tolerant to plant-parasitic nematodes (Berta *et al.*, 1990; Garcia Perez & Jaizme-Vega, 1997).

Several cases of nematodes affecting VAM-fungal development have been published. For example citrus root cortical tissue disrupted by *R. similis* effected mycelial growth and therefore suppressed the beneficial influence of this symbiont (O'Bannon & Nemec, 1979). But also here more studies are necessary to draw any conclusions.

Chapter 2: Objectives of the study

The main goal of this study was to investigate the interactions between banana, mycorrhiza and nematodes as mycorrhiza are thought to be a possible control agent against plant-parasitic nematodes.

The banana cultivar Poyo (Cavendish subgroup), the mycorrhizal strains *Glomus proliferum*, *Glomus* sp. and *Glomus intraradices* and three different populations of *Radopholus similis* were used.

In a first set of experiments, nematode-mycorrhiza interactions were studied in pot-experiments.

The first aspect was to study the effect of the mycorrhizal fungus *Glomus* sp. on the attraction, invasion and reproduction of the burrowing nematode *Radopholus similis* on banana plantlet roots and the influence of this nematode on the colonization effectiveness of the mycorrhizal strain. The two different nematode populations used were CIV, a population with a high aggressivity, and AUS, a population with a low aggressivity. The duration of this experiment was 11 weeks (experiment 1).

The second aspect was to study the effect of mycorrhization (*Glomus proliferum* and *Glomus* sp.) on the attraction and penetration of the burrowing nematode *Radopholus similis* to banana plantlet roots. Two different populations were used: a population with a low aggressivity (AUS) and a more aggressive population (CMR). This experiment was performed on a short time scale (24 and 48 hours) (experiment 2a and 2b).

In a second part of our work the penetration of nematodes in non-mycorrhizated roots was studied in more controlled conditions. Thanks to an experimental setup (experiment in Petri dish) it was possible to have a closer view on the nematode penetration in roots.

The goal of this experiment was thus to develop a controlled method to assay the initial attraction and penetration of the burrowing nematode *Radopholus similis* to banana plantlet roots (experiment 3).

Chapter 3: Materials and methods

3.1 Preparation of the experiments

Biological material

The banana cultivar used in all the experiments is Poyo cv. 902, which belongs to the *Musa* AAA group and the Cavendish subgroup. The banana plants are produced through *in vitro* micropropagation by Vitropic, Montpellier (France).
This cultivar was chosen because its susceptibility to banana nematodes, especially *Radopholus similis* (Wehunt *et al.*, 1978; Price, 1994; Pinochet *et al.*, 1998).
The plantlets were delivered in boxes of 100 units, containing 100 ml of a growth medium (MS rooting, Murashige & Skoog, 1962).
At time of delivery, the plant height was around 6 cm and weights between 0,5 and 2 grams.

Two different mycorrhiza strains were used in the experiments. They respectively belong to the species *Glomus proliferum* (given by UCL, Belgium) and *Glomus* sp. (given by Biorize®, France, code of the strain: DA). A third strain was tested for its effectivity. This strain belongs to the species *Glomus intraradices* Schenck and Smith (given by IRTA, Spain).

The nematodes that were used belong to the species *Radopholus similis* (Cobb, 1893) Thorne, 1949 and were routinely cultured monoxenically on carrot discs at CIRAD, Montpellier (France). Three different populations were used, originating from Cameroon, Ivory Coast and Australia. The original hosts for these populations are respectively *Musa* AAB, *Musa* AAA cv. *Poyo* and *Musa* AAA). The populations from Cameroon and Ivory Coast are highly pathogenic while the population from Australia has a low pathogenicity (see paragraph 1.2.2).

Growth of plant material

Preparation prior to planting involved washing the roots to remove the medium, removal of all the leaves except the 2 or 3 youngest and cutting the roots, except 2 or 3, which were then cut 1 cm from the stem.

The banana plantlets were placed individually in pots (250 ml, 7 cm diameter at the top, 10 cm height), containing an acclimatization substrate sterilized at 121 °C for 20 minutes to prevent any contamination by nematodes or VAM-fungi. The substrate consisted of 7/8 Biot sand and 1/8 soil containing 25 % dry matter, 20 % organic matter, 160-260 mg/l N, 180-280 mg/l P_2O_5, 200-350 mg/l K_2O and a pH(H_2O) 5,5-6,5. To 50 % of the banana plantlets a mycorrhizal inoculum was added. To make the mycorrhizal inoculum, leek (*Allium porrum* L.) was cultivated on a substrate (see above) containing mycorrhizal roots. After 8 weeks, the roots of the leek were washed and chopped in 0,5 – 1,0 cm pieces. These roots were homogeneously distributed in the substrate.

Once the plantlets were planted, they were watered with distilled water and the leaves were sprayed with a fine mist of distilled water. The pots were then placed for one week in a tray with a plastic cover thus creating a micro greenhouse. Two times a day, the spraying of the leaves was repeated to maintain maximum humidity during the initial weaning period. Plants were watered when needed with distilled water until the end of the experiment and no additional fertilizer was added.
During this acclimatization phase, the plants were placed in a culture room at a temperature of 25 °C, a relative humidity of 73 % and a 12 hour/12 hour photoperiod.

Nematode culture

The nematodes were produced on carrot discs (O'Bannon & Taylor, 1968). The initiation of monoxenic cultures of nematodes on carrot discs required four steps:

- preparation of the carrot discs;
- collection of nematodes from older cultures;
- sterilization of the nematodes;
- transfer of the nematodes to the *in vitro* carrot discs.

The storage roots of newly harvested carrots (*Daucus carota* L.) were washed in water, dipped in 95 % ethyl alcohol, flamed, peeled and sliced into discs of different thickness. The discs were wedged in a horizontal column across a 100 ml culture jar, 2 cm above a thin layer of 1 % water agar and Streptomycine at 500 ppm. The flasks were places in a box at 27 °C for one or two weeks.

Prior to the inoculation of the carrot discs, nematodes from older cultures were collected and surface sterilized by a method of centrifugation in a solution of $HgCl_2$ and Streptomycine (Annex 3).

Three or 4 drops of this solution were added on top of the carrot discs. The jar was closed and sealed with a plastic film to retain humidity and reduce any chance of contamination. Carrot preparation and nematode inoculation were carried out inside a laminar airflow cabinet.
Nematode cultures were incubated in the dark at 27 °C. Nematodes burrowed into the carrot tissue, fed and reproduced. After 3 to 4 weeks they migrated from the carrot discs and became clearly visible on the walls of the culture jar.

Nematode collection

Once nematodes became visible on the walls of the culture jar, each jar was checked under the binocular loupe to ascertain whether the nematodes were alive and active. After adding 2 - 3 ml of sterile water, a turn of the jar suspended the nematodes in the water. The nematode collection was then transferred to a graduated tube.
Nematode concentration was assessed by counting, and dilution carried out if necessary.

Nematode counting procedure

The nematode suspension was shaken well to ensure an even suspension. A sample was then pipetted into a counting cell and the number of nematodes per milliliter was assessed using a light microscope at x40 magnification and recorded. The average number obtained from three samples gave the estimated number of nematodes per milliliter of suspension. This solution was further diluted or concentrated to obtain the concentration that was needed for the inoculation.

Inoculation of banana plants with nematodes in pots

The chosen amount of nematodes suspended in 2 ml of sterile water was inoculated to each nematode treated plant. A hole of 1 cm diameter and 2 cm deep was made in the pots on a distance of 1 cm from the plant. The suspension was applied slowly with the use of a glass or a plastic micropipette. When a plastic micropipette was used, a few millilitres of water were applied to remove the remaining nematodes from the pipette.

With the use of a glass pipette this was not necessary. After inoculation, the holes were closed.

Inoculation of banana roots in Petri dish

Small Petri dishes (5,5 cm in diameter) were used for a new experimental setup. A more controlled condition was developed to assay the initial attraction and penetration of the nematodes in the roots a few days after inoculation.

Prior to putting the roots in the Petri dish, 15 grams of sand (Sand of Fontainebleau, diameter: 230-310 µm) was added to the Petri dish.

The root fragments to be tested had a length of 4,5 cm and consisted of either the meristem part or the non-meristem part of the root. They were placed in a Petri dish and an additional 15 grams of sand were added. Six ml of sterile water was added to saturate the sand, and the plate was covered with the cap. The dishes were put in a dark box at 27 °C for 24, 48 or 72 hours. For the time of 72 hours, the dishes were sealed with parafilm to retain humidity.

3.2 Experimental procedure

Experiment 1

The objective of this study was to investigate the effect of the mycorrhizal fungus *Glomus* sp. on the attraction, invasion and reproduction of the burrowing nematode *Radopholus similis* to banana plantlet roots and the influence of this nematode on the colonization effectiveness of the mycorrhizal strain. This experiment was performed in pot-culture; the duration of the experiment was 11 weeks.

At planting 50 % of the plants were mycorrhizated with *Glomus* sp. (Myc); the other 50 % remained non-mycorrhizated (Tem).

After 4 weeks, the plants were transferred to square pots (850 ml; 7,5 cm at the base, 10,5 cm at the top) consisting the same substrate as in the beginning of the experiment (1/8 soil, 7/8 Biot sand). Two weeks later the plants were inoculated with either a population of *Radopholus similis* from Australia (AUS) or a population from Ivory Coast (CIV). A treatment without nematodes was used in these experiments (∅).

The different treatments were:

Myc AUS	Myc CIV	Myc ∅
Tem AUS	Tem CIV	Tem ∅

Two hundred nematodes were added to each nematode treated plant. For the inoculation they were suspended in 2 ml of sterile water.

Ten replicates were made for each treatment.

After inoculation, the 60 plants (10 x 6) were placed in a completely random design to include the edge effects in the total variance.

Nematode attraction, penetration and multiplication was investigated 5 weeks after inoculation.

Mycorrhizal root colonization was assessed at the end of the experiment, 11 weeks after planting.

Experiment 2

In these experiments, also performed in pot-culture, we investigated the effect of mycorrhization (*Glomus proliferum*) on the attraction and penetration of the burrowing nematode *Radopholus similis* to banana plantlet roots. The duration of these experiments were either 24 hours or 48 hours.

Experiment 2a (24 hours)

Fifty percent of these plants were inoculated with *Glomus proliferum* (Myc), the other 50 % remained non-mycorrhizated (Tem). After 10 weeks, the plants were inoculated and again two different populations were used: a population from Australia (AUS) and a population from Cameroon (CMR 12).

The different treatments were:

Myc AUS	Tem AUS
Myc CMR 12	Tem CMR 12

Two hundred nematodes, suspended in 2 ml of sterile water were inoculated to the plants.

Five replicates were made for each treatment.

After 24 hours the roots were stained for observation (see staining procedure, paragraph 3.3).

Experiment 2b (48 hours)

The mycorrhiza strain used in this experiment was *Glomus* sp. After 9 weeks, plants were inoculated and two different populations were used (AUS, CMR 12). 400 nematodes suspended in 2 ml of sterile water were inoculated to the plants.

Six replicates were made for each treatment.

After 48 hours, the roots were stained (see staining procedure) to investigate the nematode penetration.

Mycorrhizal root colonization was assessed at the end of the experiment.

Experiment 3

In this experiment, performed in Petri dishes, only non-mycorrhizated roots were used to study the initial penetration of the nematodes in the roots. Subjects to investigate were the inoculation procedure (amount of nematodes and place of inoculation), the time interval between inoculation and observation, the segment of the root used, and the humidity of the sand.

Experiment 3a

Two different inoculum quantities and 2 different inoculum procedures were investigated after 3 time-intervals.

The different treatments were:

200 nem: 200 nematodes were inoculated on the surface of the sand, along the root;
400 nem: 400 nematodes were inoculated on the surface of the sand, along the root;
200/200 nem: 2 curves (3 mm deep) were made on both sides along the root at a distance of 2 mm from the root. The inoculum (2 times 200 nematodes) was inserted in these curves.

For these experiments root fragments including the meristem were used.

The population of nematodes used was *Radopholus similis* CMR 12.

Five replicates were made.

Nematode penetration was investigated (after staining, see staining procedure) at 3 time intervals, 24 hours, 48 hours and 72 hours after the inoculation.

Experiment 3b

In a first treatment the nematode penetration in root fragments including the meristem (mer +) was compared with the penetration in root fragments excluding the meristem (mer -). In another treatment, the impact of the humidity (hum) was tested. Respectively 5,5 ml and 6 ml of water were added to the sand in the Petri dish.

Treatments:

200/200 nem, mer - : a non-meristem part of the roots was used (the root was cut at least 4 cm from the meristem);
200/200 nem, mer + : a meristem part of the root was used;
200/200 nem, hum 5,5 ml: in stead of adding 6 ml of sterile water, only 5,5 ml was added;
200/200 nem, hum 6 ml: the usual 6 ml of sterile water was added.

For the humidity treatment the root fragment included the meristem.
The used population of nematodes is *Radopholus similis* CIV (Ivory Coast).
Eight replicates were made.
After 72 hours, the roots were stained for the observation of the nematode penetration.

Experiment 3c

The question to ask here was whether the root needs an adaptation phase after having been cut. In the treatment 'adapt 24h', the Petri dish with the root was stored for 24 hours in a box at 27°C before adding the inoculum.

Treatments:

200/200 nem, adapt 24h: Before inoculation, the Petri dish was placed in a dark box (27 °C) for 24 hours. The same amount of the evaporated water was added right before inoculation;
200/200 nem, adapt 0h: The roots were inoculated directly after the preparation of the Petri dishes.

The root fragments used in these experiments included the meristem.
The used population of nematodes is *Radopholus similis* CIV.

Eight replicates were made.
After 48 hours, the roots were stained for the observation of the nematode penetration.

Experiment 3d

The place of adding the inoculum was investigated (Figure 2.1)

Treatments:

400 nem, inoc half: The nematodes (400) were inoculated in the middle of the root (Figure 2.1a);
400 nem, inoc mer: The inoculum (400 nematodes) was all added on the meristem part of the root (Figure 2.1b).

The root fragments used in these experiments included the meristem.
The used population of nematodes is *Radopholus similis* CIV.
Eight replicates were made.
72 hours after incolation, the roots were stained for the observation of the nematode penetration.

3.3 Procedure of analysis

Harvest of plant material of experiment 1

At harvest the soil was carefully washed from the roots of the plants and plant height (pseudostem from corm base to petiole level of the youngest leaf), shoot and root fresh weight were recorded.
Roots were mixed and the nematodes were extracted following a maceration centrifugal flotation method described in annex 4. This method uses the difference in density between the nematodes and their surrounding vegetative matter to obtain their separation.
Of 5 replicates of the mycorrhiza and nematode treatments (Myc AUS and Myc CIV), 50 % weight of the roots was used to estimate the mycorrhizal colonization (see below). In the treatment Myc ∅, all the 10 replicates were used (50 % weight) for the mycorrhizal estimation.
After the extraction, the nematodes for each sample were counted and the amount of nematodes per gram of roots assessed.

Nematode and mycorrhiza staining procedure

The technique for staining the nematodes and mycorrhiza, used in experiment 2 and 3, is based on a technique developed by Baker & Gowen (1996).
With this staining procedure both nematodes and mycorrhiza can be stained together. This is necessary for investigating the inter-relationships between the two different species.

Roots washed free of soil were plunged into a hot (90 °C) 0,2 % potassium hydroxide (KOH) solution until the finest of them soften. The time required by this stage depends on sample thickness and plant species. In our case it took ± 1 hour. They were then rinsed in water (at room temperature) and transferred for 10 minutes to a 1 % HCl solution until they had lost any dark pigmentation. Following this, the roots were thoroughly rinsed in water and then placed into a hot (90 °C) staining solution for 1 hour. This staining solution is made of 50 % (v/v) lactic acid, 25 % (v/v) glycerol, 25 % (v/v) sterile water and 0,1 % (w/v) of acid fuchsin, all well mixed. The roots are then transferred to a destaining solution, which is identical to the staining solution, except that it contains no acid fuchsin. After ± 1 hour in the destaining solution, the roots were ready for observation.

Observation of the nematode penetration and estimation of the mycorrhizal root colonization

For the observation of the roots, the roots were pressed between two plastic slides. The bottom slide consisted of the bottom of a Petri dish of which the sides were removed; for the other slide a thin plastic tray was used.
A grid of 1,5 cm by 1,5 cm was made on the bottom slide to facilitate the observation of the roots by dividing the slide in segments. Thanks to this grid, the exact place of penetration of the nematodes can be determined so the penetration of the nematodes in different roots can be compared.
Nematode penetration was investigated using a light microscope at x40 magnification and the number of nematodes that penetrated in every segment of 1,5 cm of the root was counted (Plate 5 and 6). An estimation of the mycorrhizal colonization was made and the inter-relationship between the nematodes and the mycorrhiza examinated.

Mycorrhizal colonization assessment

A modified version of the grid line intersect method described by Giovanetti & Mosse (1980) and reviewed by Brundrett *et al.* (1994) was used to estimate the mycorrhizal root colonization. Cleared and stained roots, cut in pieces of 0.5 – 1 cm were randomly pressed on 9 cm diameter round slides (see above) on which a 1 cm square grid is drawn (Figure 2.2) and studied under the light microscope at x40 magnification. At each point where the root was found to intersect a gridline, an assessment of mycorrhizal colonization was made (mycorrhization or not). In this experiment, different mycorrhizal structures (hyphae, vesicles or arbuscules) were not distinguished because the formation of vesicles and arbuscules was fairly low. At least 100 intersections were studied and recorded for each root sample. The percentage of colonization was calculated by putting the number of intersection points found mycorrhizally colonized, over the total number of intersection points and multiplying by 100.

Statistical analysis

Prior to analysis, the experimental data from the pot experiment (experiment 1) were arranged in groups based on the independent variables: AUS Myc, AUS Tem, CIV Myc and CIV Tem. Thereafter, nematode populations (dependent variables) were $\log_{10}(x+1)$ transformed.

As analysis of variance (ANOVA) would be performed, its interpretations are valid only under certain assumptions. The data should therefore meet homogeneity of the variances of the different groups (Levene's test) and normal distribution of the dependent variables within groups (Normality test).

Afterwards, these groups were subjected to analysis of variance. Single-factor ANOVA was used as only the effects of one factor were investigated in this experiment. Means were compared by the Tukey's test ($P \leq 0.05$) for equal N (number of replicates) or by Spjotvoll/Stoline's test ($P \leq 0.05$) for unequal N.

All data analysis were performed with the STATISTICA® (Anonymous, 1997) package.

Chapter 4: Results

Experiment 1: The effect of the mycorrhizal fungus *Glomus* sp. on the attraction, invasion and reproduction of the burrowing nematode *Radopholus similis* and the influence of this nematode on the colonization effectiveness of the mycorrhizal strain.

In this experiment, performed in pot-culture, nematodes were inoculated to young banana plantlets. The roots of these plantlets were either colonized by a mycorrhizal fungus (*Glomus* sp.) (Myc) or left without mycorrhiza (Tem). Different *Radopholus similis* populations were used, an aggressive population originating from Ivory Coast (CIV) and a less agressive population originating from Australia (AUS).

No significant difference of plant height, shoot or root weight was observed between mycorrhizal and non-mycorrhizal plants in any treatments (Table 4.1, Annex 5).
For the total amount of nematodes in the roots and corm, the results of the treatment Tem CIV are significantly higher than the results of the treatment Myc AUS (Table 4.2). Between the other treatments, no significant difference was observed (Annex 6). The same results were found for the total number of nematodes penetrating the roots. No difference was found for the number of nematodes penetrating the corm.

The number of nematodes per gram of root was significantly higher in the roots inoculated with AUS compared with CIV. No significant difference was observed between mycorrhizated and non-mycorrhizated roots.

The reproduction rate for the CIV population in non-mycorrhizated roots was significantly higher than the other treatments (Table 4.2).

For the percentage of roots colonized by the mycorrhizal fungus, 11 weeks after inoculation of the fungus, no difference was observed between the different treatments. In roots inoculated with CIV, 42,2 % of the roots was colonized with mycorrhiza (Annex 7). In roots with AUS, a colonization of 29,7 % was observed while in the control 29,8 % of mycorrhizal colonization was present.

Experiment 2: The effect of mycorrhization (*Glomus proliferum* and *Glomus* sp.) on the attraction and penetration of the burrowing nematode *Radopholus similis* to banana plantlet roots.

In this experiment, also performed in pot-culture, the different *Radopholus similis* populations used were an aggressive population originating from Cameroon (CMR 12) and a less aggressive population originating from Australia (AUS). The nematodes were inoculated to banana plantlets which were either inoculated with a mycorrhizal fungus (*Glomus proliferum* or *Glomus* sp.) or left without mycorrhiza.

As almost no nematodes were observed for all treatments, the experiment was cut short (Annex 8). Table 4.3 gives the results observed in this experiment but a lack of data made it impossible to perform statistical analysis.

Plants inoculated with *Glomus proliferum* showed no mycorrhizal colonization while the inoculum of *Glomus* sp. was more effective, giving a colonization percentage of 34,7 % (Annex 9). This colonization consisted of a network of hyphae but almost no vesicles were observed.

An additional observation was performed in order to estimate the colonization of *Glomus intraradices* on roots of banana plants 5 weeks and 8 weeks after inoculation. After 5 weeks, 18,0 % of root colonization was observed, while after 8 weeks, this percentage went up to 44,8 % (Annex 9). Almost half of the mycorrhizal structures observed were vesicles. The hyphae were thin and almost invisible.

Experiment 3: Development of a controlled method to assay the attraction and penetration of the burrowing nematode *Radopholus similis* to banana plantlet roots.

The aim of this experiment, performed in Petri dish, was to have a first approach on the mechanisms involved in the penetration of nematodes in non-mycorrhizated roots of banana plants. For this, a small scale experiment was needed. To assay the best experimental setup some parameters were investigated: the amount of nematodes inoculated, the place of inoculation, the humidity of the sand in the Petri dish, and the fragment of the root used (including the meristem or not). Observations were made 24 hours, 48 hours and/or 72 hours after inoculation. An additional experiment performed

was to investigate whether the physiology of the root changed after the root was cut from the plant.

Almost no nematodes entered the root 24 hours after inoculation (Table 4.4). The longer the time interval between inoculation and observation, the more nematodes penetrated and almost no difference was seen between the different treatments after 72 hours.

Root fragments including meristem were compared with root fragments without meristem. It seemed that nematodes prefer to enter close to the meristem (28,7 % of penetration, compared to 6,0 % in the roots without meristem) (Annex 10).
When the sand was saturated with water, the nematode penetration was higher than when the sand was not saturated (12,8 % compared to 8,0 %).
In another experiment, the root in his Petri dish with sand was stored in a box at 27 °C for 24 hours before being inoculated. It was guessed that the root may need a latency period to recover from a possible physiological stress after being cut. In this case only 3,0 % of the inoculated nematodes penetrated compared to 32,8 % that penetrated when they were inoculated right after the root was cut (Annex 10).

When the inoculum was added in the middle of the root segment or on the meristem, 70-80 nematodes penetrated out of the 400 nematodes inoculated (around 20 %) in both treatments. Furthermore it was observed in this experiment that a high percentage of the total number of nematodes entered on the place where the root was cut. When the inoculum was added in the middle of the root segment, 49,8 % of the total penetration happened in the cut. When the inoculum was added on the meristem, 34,4 % of the nematodes penetrated in the cut (Annex 10).

Chapter 5: Discussion and conclusions

Plant growing characteristics

The very low plant height, dry shoot weight and dry root weight recorded is due to the very poor substrate used in the experiment. The substrate consisted only for 1/8 out of soil (7/8 sand) and the soil was sterilized before use. No fertilizer was added. This poor substrate was chosen to have a low level of phosphorus as mycorrhizal root development is generally inhibited by high P soils (Gerdemann, 1968; Hussey & Roncadori, 1982; Saleh & Sikora, 1984; Smith *et al.*, 1986 a, b; Smith, 1988). In very phosphorus deficient soils the percentage of mycorrhizal infection is generally lower than in soils where some phosphate is added, but at high phosphate levels mycorrhizal infections die out (Mosse, 1981).

No significant difference was observed between plant height, dry root weight and dry shoot weight in all the treatments. The first weeks after inoculation with the fungus, the fungus needs a lot of carbohydrates to develop, making those nutrients not available for the host plant. Grow of the mycorrhizated plants is thus limited. After several weeks, this phenomenon dies out, making the mycorrhizated plants growing faster than the non-mycorrhizated plants. The duration of our experiment was only 11 weeks, too short to see any difference in growth response. Another possible explanation is a maybe too low root colonization to be effective (see below).

Efficiency of the root colonization

No significant difference was observed between the number of nematodes in the roots of the mycorrhizated and the non-mycorrhizated plants. This is probably due to the short duration of the experiment but we can not exclude a possible inefficiency of the mycorrhizal strain. A colonization assessment of the mycorrhizal fungus recorded a colonization of 30 - 40 % at the moment of harvest. Nevertheless one can not neglect that there is a difference between percentage of colonization and the efficiency of the strain. Past research indicated that a mycorrhizal infection only could be effective if the colonization exceeded 20 - 30 % (Snellgrove *et al.*, 1982; Sanders *et al.*, 1977). Pinochet *et al.*, (1997) however showed a root colonization of 27 % being very effective, Cooper & Grandison (1986) observed a total absence of nematodes by a colonization of 10 %,

while Saleh & Sikora (1984) needed a colonization of 55 % to be effective (38 % colonization seemed to be ineffective).

Jaizme-Vega *et al.* (1991) recorded 38 % of the roots colonized by *Glomus fasculatum* 6 months after inoculation while Umesh *et al.* (1988) and Lin & Chang (1987) reported more than 80 % infection with the same fungus 4 months after inoculation.

In one of the experiments performed in this study *Glomus proliferum* was used. This new identified species was first observed on banana in Guadeloupe (Declerck *et al.*, 2000), confirming its ability to colonize the roots of banana. Nevertheless it seemed to be not able to colonize the banana roots in the conditions we used for the experiments. No colonization was observed.

Another experiment was carried out with the use of the mycorrhizal strain *Glomus intraradices*. Although the same conditions were used as in the experiment above, a colonization of 44.8 % was observed after 8 weeks.

These results indicate that the efficiency of a mycorrhizal strain is dependent on a lot of factors, mainly the kind of mycorrhizal strain and the environmental parameters. Each host-mycorrhiza interaction thus has its own preferable environmental conditions and they must be studied to make the mycorrhizal colonization more efficient.

The mycorrhizal structures formed by *Glomus* sp. showed a total different morphology than the structures formed by *Glomus intraradices.* With *Glomus* sp. a network of thick hyphae was found and almost no vesicles were observed (Plate 7). Colonization with *Glomus intraradices* in the same conditions consisted of many vesicles and a really thin network of hyphae (Plate 8). Pinochet *et al.* (1995) also observed many vesicles in experiments where *Glomus intraradices* was used. This morphological difference in colonizing the roots makes it difficult to compare the colonization of different species and thereby to draw any conclusions about their efficiency.

The parameter 'nematodes per gram of roots (= density)' has to be taken with care. When mycorrhizal treatments are compared with non-mycorrhizal treatments, a significant lower final nematode population is found in the mycorrhizated roots. This can be due to bigger roots systems in mycorrhizated roots, capable of supporting a larger nematode populations, i.e. dilution of the nematode root population (Jaizme-Vega *et al.*, 1997). When this is the case, a higher root weight is recorded for the mycorrhizated roots. In our

experiments however no difference was found in the root weight between the different treatments, excluding this statement.

The dynamic of multiplication of nematodes is higher than the extend of infection of mycorrhiza. Therefore the sequence in which plants are inoculated with a pathogen relative to the time of VAM-fungal inoculation may affect the nature of the interaction (Hussey & Roncadori, 1982; Smith, 1987). When plants are inoculated with mycorrhiza several weeks before being challenged with the pathogen, the mycorrhiza can colonize the root and can grow and develop freely without the influence of the pathogen. Although precolonization with VAM-fungi may represent an artificial system that favors the mycorrhizal symbiosis, it is a realistic system for containerized or transplanted hosts that can be inoculated with VAM-fungi before they are planted into field soil (Hussey & Roncadori, 1982). This inoculation method was used in many studies (Davis & Menge, 1980; Cooper & Grandison, 1986; Smith *et al.*, 1986b). The inoculation method used in the experiment performed in this study consisted of an inoculation of the VAM-fungi 4 weeks before the inoculation of the nematodes.

Difference in pathogenicity between populations of *R. similis*

Many studies performed indicated that there is a difference in pathogenicity between different populations of *Radopholus similis.* In our experiment, we used a population with high pathogenicity (CIV) and a population with low pathogenicity (AUS) (Sarah *et al.*, 1993; Fallas *et al.*, 1995; Fallas *et al.*, 1996; Hahn *et al.*, 1996). Where it comes to the number of nematodes observed per gram of roots, the results of the inoculation with CIV were significantly higher than the results of the inoculation with AUS; the reproduction rate was significantly higher for CIV. These results thus strengthen again this statement. This variability in pathogenicity may be due to divergent evolution of populations under different environmental conditions (Sarah *et al.*, 1993). Variability observed under controlled conditions could partly explain differences in damage caused in the field. For instance, the relatively high level of pathogenicity we observed for the isolate from Ivory Coast is consistent with the severe damage reported in Ivory Coast (Sarah, 1989).

Effect of nematodes on the mycorrhizal colonization

The results found in the literature concerning this subject are rather different. In several studies nematodes seemed to suppress the colonization by mycorrhiza (Smith *et al.*, 1986b; Umesh *et al.*, 1988; Pinochet *et al.*, 1996a; Jaizme-Vega & Pinochet, 1997) while in other studies no effect was recorded (Jaizme-Vega *et al.*, 1997).

In our study no statistical analysis was performed on the data of the percentage of roots colonized with VAM-fungi. We can see however that the mycorrhization was higher when CIV was present. We can believe that the plant or the mycorrhiza set up a protection mechanism against the strong invasion by nematodes. When the nematodes damage the root system very severely, we can not exclude that the root exudates maybe alter, making the roots more attractive for the mycorrhiza, so increasing the mycorrhizal colonization. This is just a first hypothesis. The exact mechanisms involved in this interaction are not yet clearly understood and thus need some more investigation.

Conclusion

Each mycorrhiza-nematode-host combination is unique, and influenced by many environmental factors (Saleh & Sikora, 1984). The mycorrhizal inoculum, even when it is the same strain, can vary with the time or with different culture practices. Also every nematode inoculum is different, regarding the number of males, females, juveniles and eggs present.

For this reason generalizations regarding such interactions between host, fungi and nematodes have to be handled with care.

No difference in plant growing characteristics could be observed after 11 weeks, showing that mycorrhiza probably need a longer time to highly colonize the roots. The very poor substrate used can also be a reason for the low colonization. A slightly more rich substrate could be helpful to get a higher mycorrhizal colonization like already mentioned in the first paragraph of this chapter. More knowledge about the suitable mycorrhiza-host couples is needed to see if the mycorrhizal strain can be really effective in the host used.

Problems with experiments in pot-culture

Experiments in pot-culture seem very realistic because they resemble more or less the situation in the field. For the attraction, penetration and reproduction experiment with the

observation 5 weeks after inoculation this experimental setup was very useful. The aim of a further experiment however was to have an approach on the mechanisms involved in the penetration of nematodes into the roots. Investigating the exact behavior of the nematodes short after being inoculated however is difficult in such experiments. The nematodes are diluted in the big volume of substrate. After 24 hours, and even after 48 hours, almost no nematodes could be found back in the roots. With this experimental setup it was thus impossible to study the initial attraction and penetration of the nematodes short after their inoculation.

Experiments conducted in more controlled conditions

To overcome the problem described in the paragraph above a new experimental setup was developed. Experiments were conducted in Petri dishes to develop more controlled conditions. The exact place of inoculation could be chosen and then after observation, the exact pattern the nematodes had followed could be assessed. This experimental setup may represent an artificial system, compared with the more realistic pot-experiment but it was very useful to study the initial penetration of nematodes into roots of banana.

Different inoculation procedures were tested. After 24 hours, a difference could be found between the different treatments. When 400 nematodes were inoculated, in proportion more nematodes penetrated the roots than when only 200 nematodes were inoculated. After 48 hours, and certainly after 72 hours, this difference died out. Between the different inoculation procedures, the inoculation with 2 times 200 nematodes (200/200 nem) seemed to be most attractive. In this inoculation procedure, 2 curves were made in the sand on both sides along the roots and the inoculum was added in these curves. This probably prevented the inoculum to spread out, contrarily to what happened with the other inoculation procedures.

In another experiment, the effect of the humidity of the sand was tested. When the sand was saturated, the nematodes were able to move freely; a high number of nematodes penetrated the roots. When the sand was drier, the nematodes seemed to be blocked in their movements thus less nematodes penetrated.

Harley & Smith (1983) postulated that nematodes prefer to enter at or near the root tip. This statement was investigated by comparing the penetration of nematodes in 2 different segments of the root, one including the meristem, the other without meristem. Results

confirmed this statement. Much more nematodes penetrated in the root fragment including the meristem compared with the root fragment excluding the meristem.
In all those experiments above, there was a trend that nematodes have an attraction to the place where the root was cut, as Kaplan & Davis (1991) already observed.
Therefore another experiment was conducted to study the exact pattern followed by the nematodes. The inoculum was either added halfway the root or on the meristem, far away from the place were the root was cut. In both treatments there was a clear tendency that nematodes prefer to enter via the cut (Plate 9). This is a proof that this setup is artificial and has to be improved when willing to be useful for further experiments.
In a last experiment, the physiological change of the root after being cut was investigated. When the root (in the Petri dish) was stored in a box at 27 °C for 24 hours, the physiological state of the root changed. Nematodes inoculated to this root did not want to penetrate. Only a few nematodes entered the root. This is a second proof that the artificial setup in this condition does not resemble the real physiological state of the root.

Future prospects

The use of VAM-fungi as a control agent against plant-parasitic nematodes is a subject many researchers are working on. The results obtained until now are promising. However the research on this subject is still in its infancy and many more domains need to be clearly investigated.
To make the mycorrhizal colonization as efficient as possible, micropropagated plants can already been inoculated *in vitro*. Potential advantages are numerous. First, the mycorrhiza are already established in the roots and, consequently, will have a competitive advantage over soilborne pathogens. Second, higher plant growth rates may result in a shorter nursery phase. Third, far less mycorrhizal fungi propagules are required to inoculate young plantlets (having fewer roots) than older plants and may result in reduced costs (Delvaux *et al*, 1998). To be sure that the inoculum used is free of pathogens, it is safer to use VAM species originating from banana roots in monoxenic conditions (Declerck *et al*., 1998).

To have a better understanding in how mycorrhiza can have an influence on the penetration of nematodes in the roots of banana, more controlled conditions were developed (Petri dish). This experimental setup made it possible to study the exact pattern

nematodes follow after being inoculated to the roots. The idea of developing more controlled conditions to study the initial penetration of nematodes into the roots is promising. Another experimental setup however is needed to have a more precise observation without bias. The use of bigger Petri dishes, which can store a whole plantlet can already be a first step in improving this setup. In this Petri dish, different partitions can be made to separate the roots from each other. Nematodes inoculated on one root thus can not reach another root. Like this the exact conditions where nematodes penetrate a root or not can be assessed.

The study of mycorrhizal methods for disease control may uncover new mechanisms of host tolerance or resistance to nematodes and new inhibitory compounds to root infecting pathogens. These compounds can possibly be induced chemically once the biochemical mechanisms are known. Evidence from laboratory and greenhouse trials suggests that mycorrhizal fungi could play a realistic role in nematode control, but knowledge of how these fungi function in natural field conditions is required before their impact on crop protection can be assessed.

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Annexes

Annex 1: Classification of the section of *Eumusa* with some examples (Jones, 2000)

Parents	level of ploidie	genomic group	subgroup	name of clone*
	Diploid	AA		Sucrier
				Pisang Lilin
				Inarnibal
				Senorita
				Pisang Jari Buaya
				Paka
		AB		Ney Poovan
				Kunnan
				Sukari Ndizi
	Triploid	AAA	Gros Michel	Gros Michel
				Cocos
				Highgate
				Lowgate
Musa acuminata Colla AA			Cavendish	Extra-Dwarf Cavendish
				Dwarf Cavendish
				Giant Cavendish
				Poyo
				Grand Nain
				Pisang masak Hijau
Musa balbisiana Colla BB			Lujugira-Mutika	Beer
				Musakala
				Nakabululu
				Nakitembe
				Nfuuka
			other	Red and Green Red
				Lakatan
				Ibota Bota
		AAB	Plantain	French
				French Horn
				False Horn
				Horn
			Pome	Pome
				Prata Anã
				Pacovan
				Pachanadan
			Maia Maoli - Popoulu Iholena	Maia Maoli
				Popoulu
			other	Silk
				Pisang Raja
				Pisang Kelat
		ABB		Bluggoe
				Silver Bloggoe
				Monthan
				Dwarf Bluggoe
				Pisang Awak
				Pelipita
				Ney Mannan
				Kalapua
				Kluai Teparot
	Tetraploid	AAAA		Bodies Altafort
		ABBB		Klue teparod

* The 'best-known synonym' gives its name to the clone.

Annex 2: General outline presenting the main soils used for growing bananas and plantains, defined at the order level according to soil taxonomy (Soil Survey Staff, 1989) and some of their chief properties and associated soil constraints (Delvaux, 1995)

Soil order	Area	Main soil constraints	Key management practices
Histosols	Surinam, Ivory Coast	Waterlogging	Drainage
Oxisols	Africa, Asia, South America	Acidity, low CEC and nutrient status	Liming, split fertilization
Utisols	Africa, Asia, South America	High clay content, compaction, low nutrient status	Soil tillage, split fertilization, drainage
Aridisols	North America, Near East	Calcic and/or sodic features	Irrigation and drainage
Inceptisols	Africa, Asia, Central and South America, Caribbean area	Variable	Variable
Andisols	Africa, Central and South America, Caribbean area	Variable, low macroporosity when tilled, high nutrient leaching	Variable, split fertilization
Vertisols	Somalia, Central America	High content of swelling clays	Irrigation, drainage, soil tillage
Entisols	Africa, Central and South America	Texture with low CEC and water retention	Irrigation, split fertilization, drainage

Annex 3: Inoculation of the carrot jars

- Recuperate the nematodes from the jars with a Pasteur pipette with sterile water
- Empty the content of the pipette in a conic tube
- Centrifuge for 3 minutes at 2500 rpm
- Remove the supernatant and replace it by a solution of $HgCl_2$ (0.01 %)
- Homogenize and centrifuge for 3 minutes at 2500 rpm
- Remove the supernatant and replace it by sterile water
- Homogenize and centrifuge for 3 minutes at 2500 rpm
- Remove the supernatant and replace it by a solution of dihydrostreptomycine (0.2 %)
- Homogenize and centrifuge for 3 minutes at 2500 rpm
- Remove the supernatant and replace it by sterile water
- Homogenize and centrifuge for 3 minutes at 2500 rpm
- Reduce the volume to have a concentrated suspension of nematodes
- Inoculate several drops of this suspension on the carrot discs in de jars
- Place them in a box in the dark at 27 °C

Annex 4 : Maceration centrifugal flotation nematode extraction (adapted method from Coolen and D'Herde (1972))

- Place the roots in a sieve (1 mm) and wash them with water to remove soil particles
- Chop the roots in small pieces (<1 cm)
- Place the roots in a kitchen blender and add 50 ml of water
- Blend the roots at maximum speed twice during 10 seconds with an interval of 5 seconds
- Pour the blended roots in a series of sieves (from base to top: 32 µm, 50 µm, 80 µm, 250 µm) and wet sieve for 1 to 2 minutes with a strong water jet
- Remove the 250 µm sieve
- Collect the nematodes in the 80 µm, 50 µm and 32 µm sieve in a centrifugation tube (250 ml)
- Add 5g of kaolin and water up to a final volume of 200 ml
- Homogenize the solution with a mechanical stirrer
- Centrifugate at 3000 rpm for 5 minutes
- Remove the supernatant
- Pour magnesium sulfate (35% w/v) up to a volume of 200 ml
- Resuspend the residue with a mechanical stirrer
- Centrifugate at 3000 rpm for 5 minutes
- Pour the supernatant into a 5 µm sieve and recycle the magnesium sulfate
- Collect the nematodes from the sieve and put them in a test tube

Annex 5: Growth response of 'Poyo' banana to inoculation with *Glomus* sp. and two different populations of *Radopholus similis* 11 weeks after inoculation with the VAM-fungus and 5 weeks after inoculation with 200 nematodes per plant

Treatment		Replicate	Plant height (cm)	Fresh shoot weight (g)	Fresh root weight (g)
AUS	Myc	1	4.5	4,73	7,17
		2	6,8	6,12	9,16
		3	5,8	5,59	6,14
		4	5,4	3,00	6,68
		5	6,3	5,49	3,87
		6	5,8	4,99	4,78
		7	5,9	4,49	3,70
		8	5,1	4,58	3,30
		9	6,4	4,44	4,77
		10	4,8	4,69	3,43
		Mean	**5,2**	**4,81**	**5,30**
	Tem	1	6,5	7,53	4,15
		2	6,2	5,94	7,15
		3	6,7	6,54	5,25
		4	5,6	5,67	4,39
		5	5,8	5,75	5,13
		6	5,5	5,99	5,72
		7	5,9	5,17	5,34
		8	5,6	5,39	2,55
		9	5,4	4,41	4,82
		10	5,8	4,58	3,46
		Mean	**5,9**	**5,70**	**4,80**
CIV	Myc	1	6,8	8,14	7,99
		2	6,2	7,14	6,82
		3	6,0	6,32	4,80
		4	5,6	5,12	2,56
		5	5,8	6,55	3,49
		6	5,8	5,49	7,21
		7	5,4	4,63	4,66
		8	5,9	4,70	4,36
		9	5,3	4,32	3,85
		10	5,8	4,97	2,00
		Mean	**5,9**	**5,74**	**4,77**
	Tem	1	7,0	7,70	5,10
		2	7,0	7,00	6,40
		3	6,1	5,42	5,00
		4	6,0	6,10	5,10
		5	6,2	4,90	4,20
		6	6,3	6,73	3,25
		7	6,5	6,10	6,30
		8	5,5	5,50	4,30
		9	6,2	4,60	3,90
		10	5,8	4,30	4,10
		Mean	**6,3**	**5,84**	**4,77**

Treatment		Replicate	Plant height (cm)	Fresh shoot weight (g)	Fresh root weight (g)
Control	Myc	1	6,4	6,23	4,00
		2	6,8	6,87	6,69
		3	6,9	7,21	5,75
		4	5,7	6,29	4,99
		5	6,4	6,58	6,23
		6	6,8	5,99	4,00
		7	6,2	6,17	4,88
		8	5,2	4,63	4,29
		9	5,6	5,26	3,99
		10	5,0	4,60	3,50
		Mean	**6,1**	**5,98**	**4,83**
	Tem	1	6,6	7,66	8,69
		2	7,2	8,83	7,73
		3	5,8	6,18	4,69
		4	6,4	5,34	4,27
		5	5,8	5,68	4,39
		6	5,7	4,98	3,01
		7	5,5	5,69	3,60
		8	6,5	6,08	4,49
		9	5,6	5,00	3,99
		10	5,0	3,67	3,00
		Mean	**6,0**	**5,91**	**4,79**

Annex 6: Reproduction of two different *Radopholus similis* popualtions in 'Poyo' banana alone and in combination with *Glomus* sp. 11 weeks after inoculation with the VAM-fungus and 5 weeks after inoculation with 200 nematodes per plant

Treatment		Replicate	Fresh root weight (g)*	Total nematodes in roots**	Nematodes /g roots**
AUS	Myc	1	7,17	445	62,1
		2	9,16	1560	170,3
		3	6,14	1835	298,9
		4	6,68	980	146,7
		5	3,87	1005	259,7
		6	2,29	350	152,8
		7	1,62	285	175,9
		8	1,30	195	150,0
		9	2,28	25	11,0
		10	1,59	205	128,9
		Mean	**4,21**	**688,5**	**155,6**
	Tem	1	4,15	1035	249,4
		2	7,15	890	124,5
		3	5,25	710	135,2
		4	4,39	555	126,4
		5	5,13	980	191,0
		6	5,72	1480	258,7
		7	5,34	1180	221,0
		8	2,55	415	162,7
		9	4,82	743	154,0
		10	3,46	635	183,5
		Mean	**4,80**	**862,3**	**180,7**
CIV	Myc	1	7,99	2105	263,5
		2	6,82	1695	248,5
		3	4,80	2445	509,4
		4	2,56	1020	398,4
		5	3,49	2810	805,2
		6	3,35	1185	353,7
		7	2,00	1000	500,0
		8	2,03	620	305,4
		9	1,25	185	148,0
		10	1,11	323	290,5
		Mean	**3,54**	**1338,8**	**382,3**
	Tem	1	5,10	1763	345,6
		2	6,40	5720	893,8
		3	5,00	1625	325,0
		4	5,10	2255	442,2
		5	4,20	1970	469,0
		6	3,25	1005	309,2
		7	6,30	840	133,3
		8	4,30	1470	341,9
		9	3,90	58	14,9
		10	4,10	2654	647,3
		Mean	**4,77**	**1936,0**	**392,2**

* From the treatments Myc AUS and Myc CIV 50 % of the root weight of replicates 6 – 10 is taken to measure the mycorrhizal root colonization

** The means of these data can differ from the means in table 4.2 because of outliers

Treatment		Replicate	Total nematodes in roots**	Total nematodes in corm**	Total nematodes (roots+corm)**
AUS	Myc	1	445	24	469
		2	1560	110	1670
		3	1835	39	1874
		4	980	103	1083
		5	1005	20	1025
		6	350	89	439
		7	285	46	331
		8	195	31	226
		9	25	7	32
		10	205	67	272
		Mean	**688,5**	**53,6**	**742,1**
	Tem	1	1035	100	1135
		2	890	26	916
		3	710	28	738
		4	555	194	749
		5	980	122	1102
		6	1480	32	1512
		7	1180	161	1341
		8	415	36	451
		9	743	320	1063
		10	635	35	670
		Mean	**862,3**	**105,4**	**967,7**
CIV	Myc	1	2105	67	2172
		2	1695	158	1853
		3	2445	213	2658
		4	1020	22	1042
		5	2810	9	2819
		6	1185	345	1530
		7	1000	95	1095
		8	620	61	681
		9	185	22	207
		10	323	324	647
		Mean	**1338,8**	**131,6**	**1470,4**
	Tem	1	1763	104	1867
		2	5720	81	5801
		3	1625	578	2203
		4	2255	263	2518
		5	1970	71	2041
		6	1005	159	1164
		7	840	216	1056
		8	1470	136	1606
		9	58	59	117
		10	2654	32	2686
		Mean	**1936,0**	**169,9**	**2105,9**

** The means of these data can differ from the means in table 4.2 because of outliers

Annex 7: Root colonization of *Glomus* sp. in banana roots in presence or absence of two different *Radopholus similis* populations 11 weeks after inoculation of the fungus

Treatment	Replicate	Amount of intersections mycorrhizated*	% Colonization
CIV	1	55/174	31,6%
	2	60/124	48,4%
	3	88/148	59,5%
	4	58/120	48,3%
	5	26/114	22,8%
	Sum	287/680	42,2%
	Mean	**57/136**	**42,2%**
AUS	1	59/119	49,6%
	2	58/116	50,0%
	3	26/127	20,5%
	4	34/156	21,8%
	5	11/114	9,6%
	Sum	188/632	29,7%
	Mean	**38/126**	**29,7%**
Control	1	21/152	13,8%
	2	9/159	5,7%
	3	41/140	29,3%
	4	41/130	31,5%
	5	54/137	39,4%
	6	2/108	1,8%
	7	45/115	39,1%
	8	59/135	43,7%
	9	68/121	56,2%
	10	54/124	43,5%
	Sum	394/1321	29,8%
	Mean	**39/132**	**29,8%**

* The number after the slash means the amount of intersections on the slide; the number before the slash is the amount of intersections where a mycorrhizal structure could be observed

Annex 8: Penetration of two different *Radopholus similis* populations in banana roots, alone or in combination with *Glomus* sp. respectively 24 hours and 48 hours after inoculation of the nematodes

Treatment		Replicate	24h/200 nem	48h/400 nem
AUS	Myc	1	10	14,5
		2	0,5	3
		3	0	7
		4	0	~
		Mean	**2,6**	**8,2**
		% penetration	**1,3%**	**2,1%**
	Tem	1	8,5	10
		2	0	~
		3	5	~
		4	4	~
		Mean	**4,4**	**10**
		% penetration	**2,2%**	**2,5%**
CMR 12	Myc	1	1,5	9,5
		2	~	1,5
		3	~	6
		Mean	**1,5**	**5,7**
		% penetration	**0,8%**	**1,4%**
	Tem	1	2,5	4
		2	24	13,5
		3	~	6
		Mean	**13,3**	**7,8**
		% penetration	**6,7%**	**2,0%**

~ Replicate not observed because of the low number of nematodes that penetrated the roots in the already observed replicates

Annex 9: Root colonization of respectively *Glomus* sp. and *Glomus* intraradices in banana roots in absence of nematodes respectively 10 weeks and 8 weeks after inoculation

***Glomus* sp.**

Replicate	amount of intersections mycorrhizated*	% colonization
1	30/58	51,7%
2	18/61	29,5%
3	19/57	33,3%
4	18/61	29,5%
5	17/46	37,0%
6	14/51	27,5%
Sum	116/334	34,7%
Mean	**19/56**	**34,7%**

Glomus intraradices

Replicate	amount of intersections mycorrhizated*	% colonization	% vesicles
1	45/72	62,5%	58,0%
2	60/120	50,0%	33,0%
3	40/74	54,0%	67,5%
4	45/115	39,0%	55,5%
5	45/144	31,3%	50,0%
Sum	235/525	44,8%	52,8%
Mean	**47/105**	**44,8%**	**52,8%**

Annex 10: Results of the different treatments performed in the experiments in Petri dish (A) amount of nematodes inoculated observed after different time intervals (B) effect of included meristem, humidity or adaptation phase (C) effect of the place of inoculation

A

Replicate	200 nem (CMR 12)			400 nem (CMR 12)			200/200 nem (CMR 12)		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
1	0	2	40	14	0	36	171	15	30
2	0	0	178	16	2	92	41	100	117
3	0	1	48	1	80	43	45	8	76
4	26	0	20	9	35	22	0	226	105
5	4	0	42	1	35	153	8	22	88
Total	**30**	**3**	**328**	**41**	**152**	**346**	**265**	**371**	**416**
Mean	**6**	**0,6**	**65,6**	**8,2**	**30,4**	**69,2**	**53**	**74,2**	**83,2**
Penetration%	**3,0%**	**0,3%**	**32,8%**	**2,1%**	**7,6%**	**17,3%**	**13,3%**	**18,6%**	**20,8%**

B

Replicate	200/200 nem (CIV), observation 72h				200/200 nem (CIV), observ. 48h	
	mer* -	mer +	hum* 5,5 ml	hum 6 ml	adapt* 0h	adapt 24h
1	70	0	23	0	119	5
2	3	207	53	0	101	5
3	4	175	32	44	156	2
4	1	92	12	123	205	46
5	15	202	4	~	28	1
6	55	128	1	2	156	19
7	39	0	67	58	36	19
8	6	113	65	132	248	0
Total	**193**	**917**	**257**	**359**	**1049**	**97**
Mean	**24,1**	**114,6**	**32,1**	**51,3**	**131,1**	**12,1**
Penetration%	**6,0%**	**28,7%**	**8,0%**	**12,8%**	**32,8%**	**3,0%**

C

Replicate	Segment*	400 nem (CIV), observ. 72h	
		Inoc. half	Inoc. meristem
1	A1	3	9
	A2	13	6
	A3	4	2
	cut	28	17
2	A1	21	16
	A2	17	10
	A3	21	13
	cut	77	15
3	A1	52	89
	A2	54	17
	A3	0	6
	cut	24	2
4	A1	0	6
	A2	7	8
	A3	2	15
	cut	42	5
5	A1	7	11
	A2	2	49
	A3	2	13
	cut	0	50
6	A1	1	27
	A2	3	9
	A3	9	5
	cut	45	20
7	A1	~	51
	A2	~	5
	A3	~	20
	cut	~	24
8	A1	~	6
	A2	~	12
	A3	~	5
	cut	~	82
Total		**434**	**625**
Mean		**72,3**	**78,1**
Penetration%		**18,1%**	**19,5%**
% on cut		**49,8%**	**34,4%**