Katholieke Universiteit Leuven

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Studie van de interacties tussen endomycorrhizae en *Radopholus similis* populaties die verschillen in pathogeniciteit, in de wortels van *Musa* spp.

(Study of the interactions between endomycorrhizae and *Radopholus similis* populations with a difference in pathogenicity, in the roots of *Musa* spp.)

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Samenvatting

Inleiding

Bananen vormen het basisvoedsel voor een belangrijk deel van de wereldbevolking. Slechts 15 % van de wereldproduktie van bananen wordt geëxporteerd; deze bananen worden geproduceerd in commerciële plantages. De overige 85 % wordt vooral door kleine boeren verbouwd voor lokale consumptie.
De teelt van bananen wordt echter bedreigd door talrijke ziekten en plagen, oa. veroorzaakt door plant-parasitaire nematoden. Tot de belangrijkste nematodenspecies die bananen infecteren, behoren *Radopholus similis* (Cobb) Thorne, *Pratylenchus goodeyi* en *P. coffeae* Sher en Allen en *Helicotylenchus multicinctus* (Cobb) Golden, allen migratorisch endoparasitair. Dit betekent dat ze hun levenscyclus volledig volbrengen in de gastplant, maar zich vrij kunnen verplaatsen tussen de wortels van de plant en de bodem. Deze nematoden voeden zich met het cytoplasma van de wortel- en cormuscellen, waardoor gaatjes ontstaan in het weefsel hiervan. Deze necrosis is zichtbaar als een rood-bruine verkleuring van de cellen. Als gevolg hiervan wordt de opname en het transport van water en nutriënten zwaar belemmerd, hetgeen leidt tot een verminderde groei van de plant en een verzwakking van de mechanische verankering in de bodem. Hierdoor ontstaat de kans dat de planten omvallen bij hevige wind of door het gewicht van hun eigen vruchten. De schade die nematoden aanrichten aan bananen wordt geschat op 20 % van de jaarlijkse produktie op wereldschaal.

Cultuurmaatregelen voor de bestrijding van nematoden zijn meestal gebaseerd op chemische controle. Chemische bestrijding door middel van nematiciden is echter enkel economisch haalbaar in grote plantages omwille van de hoge kosten. Daarbuiten brengen nematiciden een zwaar ecologisch risico met zich mee. Een interessante toepassing voor de bestrijding van plant-parasitaire nematoden is de controle door biologische antagonisten.

Tijdens de laatste decennia zijn er meerdere studies uitgevoerd over het gebruik van arbusculaire mycorrhizae (AM-fungi) als een mogelijk biologisch controle agens. AM-fungi zijn goed gekend voor het verbeteren van de kwaliteit van de plant door een verhoogde opname van water en nutriënten. Na de kolonisatie van de plant door de fungus groeit nog een deel van de hyfen buiten de wortel. Deze hyfen vormen een

netwerk rond de wortels, wat het absorberende oppervlak van de wortels aanzienlijk vergroot. Tevens verhogen ze de toegang voor de plant tot fosfaatbronnen in de bodem aangezien ze beduidend effectief zijn in het absorberen van fosfaten. Door de verhoogde sterkte van de plant, als gevolg van de aanwezigheid van mycorrhizae, wordt de natuurlijke resistentie tegen biotische (pathogenen) en abiotische (waterstress, vervuiling, zout) onevenwichten aanzienlijk verbeterd.

De mutualistische symbiose tussen AM-fungi en bananenplanten is niet gast-specifiek. Een enkele gastplant kan associeren met de meeste mycorrhiza species, terwijl een enkele fungus een hele reeks van gastplanten kan infecteren. Onderzoek heeft echter aangewezen dat de efficiëntie van de symbiose afhangt van het fungus/gastheer koppel en de daarbij horende omgevingscondities. Daarbij komt nog eens dat elke gastheer/fungus/nematode combinatie uniek is, wat van deze toepassing een onderwerp maakt van zeer grote complexiteit.

Doelstelling van het onderzoek

Verschillende experimenten werden ontwikkeld om beter inzicht te verkrijgen in de drie stappen die tussenkomen in de interactie tussen AM-fungi en nematoden. De aantrekking, penetratie en reproduktie van de nematoden naar de wortels van bananenplanten werden onderzocht in aan- en afwezigheid van AM-fungi.

In een eerste groep van experimenten werd de interactie tussen nematoden en mycorrhiza bestudeerd in potcultuur. Daarbij werd in een eerste experiment het effect nagegaan van AM-fungi op de penetratie en reproduktie van verschillende *R. similis* populaties in de wortels van jonge bananenplanten. Ook werd de invloed van deze nematoden op de effectiviteit van de kolonisatie van de AM-fungus bestudeerd. Twee verschillende nematodenpopulaties met een verschil in pathogeniciteit werden hiervoor gebruikt.
Aangezien in twee erkende laboratoria twee verschillende methoden gebruikt worden voor de inoculatie van mycorrhiza op de wortels van de bananenplanten, werd een bijkomend experiment opgezet ter vergelijking van deze twee methoden. Het doel van het experiment was te bestuderen of er tussen de twee gebruikte methoden verschillen optraden in de wortelkolonisatie door de fungus en of er een effect was van deze kolonisatie op de groei en wortelontwikkeling van de planten. Aangezien *in vitro* aangemaakte plantjes geïnoculeerd moeten worden met AM-fungi alvorens overgeplant

te worden naar het veld is het belangrijk een procedure toe te passen die een snelle kolonizatie door de fungus toelaat. De plantjes die reeds snel voordelen kunnen halen uit de symbiose met AM-fungi geven zowel economisch voordeel als een betere tolerantie tegen plant-parasitaire nematoden.
Het is nog niet volledig duidelijk of AM-fungi tussenkomen in de aantrekking en penetratie of in de reproduktie van nematoden in de wortels. Daarom werd een volgend experiment opgezet waarin enkel de aantrekking en penetratie van nematoden werd bestudeerd in de wortels van bananenplanten in aan- of afwezigheid van AM-fungi.

In een tweede reeks experimenten werd de aantrekking en penetratie van nematoden bestudeerd in meer gecontroleerde condities. Deze experimenten werden uitgevoerd in Petriplaten. Een eerste proefopzet bestond uit Petriplaten, gevuld met zand, waarin een wortelsegment werd ingebracht. Op deze manier kon de exacte plaats van inoculatie van de nematoden gekozen worden en kon de exacte plaats waar de nematoden in de wortel penetreerden bepaald worden. Het doel van dit experiment was na te gaan of de nematoden in de wortel penetreren op de plaats waar ze geïnoculeerd werden, of dat ze langs de wortel bewegen op zoek naar de meest geschikte plaats om in de wortel te penetreren.
Tenslotte werd een *in vitro* experiment opgezet om de aantrekking van nematoden naar de wortel te bestuderen. Het spoor van de nematoden kon gevolgd worden vanaf het moment dat ze geïnoculeerd werden tot op het moment dat ze penetreerden in de wortel. In dit experiment kon echter enkel de interactie tussen verschillende bananencultivars en verschillende nematode populaties bestudeerd worden, aangezien tot op heden *in vitro* gemycorrhizeerde planten nog niet bekomen kunnen worden. Het doel van dit experiment was na te gaan of er een verschil is in aantrekking naar de wortels tussen nematode populaties met een verschillende pathogeniciteit, en of twee verschillende bananencultivars een verschillende invloed hebben op de aantrekking van de nematoden naar de wortels.

Resultaten en discussie

Deel I: Experimenten uitgevoerd in potcultuur

Experiment 1: Interactie tussen *Glomus* sp. en *Radopholus similis* bij de *Musa* cultivar Poyo

Het doel van dit experiment was het bestuderen van het effect van de mycorrhiza species *Glomus* sp. op de penetratie en reproduktie van de nematode *R. similis* bij de *Musa* cultivar Poyo. Dit experiment werd uitgevoerd in potcultuur. De mycorrhizae werden geïnoculeerd op de bananenplantjes en 4 weken later werden de plantjes geïnoculeerd met nematoden. Twee verschillende *R. similis* populaties werden hiervoor gebruikt: de sterk pathogene populatie afkomstig uit Ivoorkust en de weinig pathogene populatie afkomstig uit Australië.

Er kon geen verschil waargenomen worden in planthoogte, scheutgewicht en wortelgewicht tussen de verschillende behandelingen, 11 weken na de inoculatie met de mycorrhiza (Tabel 5.1 pg 63). Opvallend is ook dat deze groeikarakteristieken zeer laag waren voor de duur van het experiment. Dit is waarschijnlijk te wijten aan het zeer arme substraat dat gebruikt werd in het experiment. Het substraat bestond voor 7/8 uit zand en voor 1/8 uit grond die vooraf geautoclaveerd was. Er werden ook geen groeiverbeteraars toegevoegd. Dit zeer arme substraat was gekozen om de ontwikkeling van de mycorrhizae te bevorderen daar het geweten is dat deze ontwikkeling geremd wordt als het gehalte aan P in het substraat te hoog is (Gerdemann, 1968; Hussey & Roncadori, 1982; Smith *et al.*, 1986 a, 1986 b; Smith, 1988). Een reden waarom er geen significante verschillen optraden tussen de behandelingen met en zonder mycorrhiza kan te maken hebben met de korte duur van het experiment. Waarschijnlijk was een periode van 11 weken niet voldoende om de plant optimaal voordeel te kunnen laten halen uit de symbiose. Vlak na de infectie door de fungus komt ongeveer 10 % van de C die door de plant wordt doorgegeven naar de wortels terecht bij de fungus, zodat deze C niet meer beschikbaar is voor de plant. Na enkele weken, wanneer de fungus reeds verder ontwikkeld is wordt de C-balans hersteld: de C die doorgegeven wordt naar de fungus wordt dan gecompenseerd door de verhoogde fotosynthese ten gevolge van de verhoogde P-status, bekomen dankzij de aanwezigheid van de fungus. Vanaf dat moment ondervindt de plant voordeel van de symbiose en wordt de groei bevorderd. Als gevolg hiervan wordt de eerste weken na de associatie tussen gastplant en fungus een groeireductie van

de gemycorrhizeerde plant waargenomen, die achteraf zal verdwijnen. Aangezien er geen verschil in groeikarakteristieken kon waargenomen worden tussen de gemycorrhizeerde en de niet-gemycorrhizeerde planten in dit experiment kan er aangenomen worden dat de duur van het experiment juist voldoende was voor de planten om het stadium van de groeireductie te verlaten, maar dat de tijd te kort was om reeds voordeel te kunnen halen uit de symbiose.

Het is ook mogelijk dat een wortelkolonisatie van 30 tot 42 % (Tabel 5.3 pg 65) niet voldoende was om een effectieve symbiose tot stand te brengen daar er vaak geen rechtlijnig verband bestaat tussen colonizatie frequentie en effectiviteit van de symbiose (Carroll *et al.*, 1985; Smith *et al.*, 1992) of dat het niet waarschijnlijk is dat een sterk verbeterde groei kan verwacht worden met de cultivar Poyo wegens zijn lage mycorrhiza-afhankelijkheid (Declerck *et al.*, 1995).

Voor het totaal aantal nematoden dat teruggevonden werd in de wortels kon er een significant ($P \leq 0.05$) verschil waargenomen worden tussen de twee *R. similis* populaties (Tabel 5.2 pg 64). Van de pathogene populatie, afkomstig uit Ivoorkust werden meer nematoden teruggevonden in de wortels, vergeleken met de weinig pathogene populatie afkomstig uit Australië. Ook werden er significant ($P \leq 0.05$) minder nematoden teruggevonden in de gemycorrhizeerde planten vergeleken met de niet-gemycorrhizeerde planten. Dit bevestigt het voordelig effect dat AM-fungi kunnen hebben op de onderdrukking van nematodenpopulaties in de wortels. De invloed van de AM-fungi was meer uitgesproken wanneer het ging om de sterk pathogene nematodepopulatie. Dezelfde significante verschillen konden worden waargenomen betreffende de graad van reproduktie.

Er zijn reeds veel studies uitgevoerd over het verschil in pathogeniciteit tussen verschillende populaties van *R. similis* (Sarah *et al.*, 1993; Fallas *et al.*, 1995; Fallas *et al.*, 1996; Hahn *et al.*, 1996). De variabiliteit in pathogeniciteit blijkt te wijten te zijn aan een divergente evolutie onder verschillende omgevingscondities. De resultaten uit voorliggend experiment bevestigen de conclusies van meerdere onderzoekers, namelijk dat de populatie afkomstig uit Ivoorkust een hogere pathogeniciteit heeft dan de populatie komende van Australië.

Wanneer we keken naar het effect dat de nematoden hadden op de wortelkolonisatie konden we vaststellen dat er een trend was naar een hogere wortelkolonisatie in aanwezigheid van de sterk pathogene populatie (Ivoorkust) vergeleken met de weinig pathogene populatie (Australië). Deze verschillen waren echter niet significant. Verschillende studies hebben aangetoond dat nematoden een onderdrukkend effect kunnen hebben op de wortelkolonisatie door de fungus (Smith *et al.*, 1986b; Umesh *et*

al., 1988; Pinochet *et al.*, 1995; Jaizme-Vega *et al.*, 1997) of dat er geen invloed vastgesteld kon worden van de aanwezigheid van nematoden op de wortelkolonisatie (Jaizme-Vega *et al.*, 1997). De oorzaken van een onderdrukkend effect kunnen te wijten zijn aan een competitie voor ruimte of voor koolstofbronnen tussen de fungus en de nematoden. In dit experiment werd echter een bevorderend effect waargenomen van de nematoden op de wortelkolonisatie door de fungus. Het is mogelijk dat de nematoden een verandering in de wortelexudaten, uitgescheiden door de plant veroorzaakten na penetratie in de wortel, en dat deze verandering de condities voor de ontwikkeling van de fungus meer aantrekkelijk maakte. De precieze mechanismen die tussenkomen in de interactie tussen nematoden en mycorrhiza zijn echter nog niet gekend, dus meer onderzoek is nodig alvorens conclusies te kunnen formuleren over dit onderwerp.

Experiment 2: Vergelijking van twee methoden voor de inoculatie van mycorrhiza

Het doel van dit experiment was na te gaan of er een verschil ontstaat in groeikarakteristieken tussen planten die groeien op een substraat waar de mycorrhiza zijn gehomogeniseerd met het substraat en planten die groeien op een substraat waaraan de mycorrhiza zijn toegevoegd in een laagje van inoculum gelegen tussen twee lagen van substraat. Ook werd de wortelkolonisatie door de fungus bestudeerd in de twee behandelingen.

Er konden significante verschillen waargenomen worden in planthoogte ($P \leq 0.01$) en wortelgewicht ($P \leq 0.05$), enerzijds tussen de gemycorrhizeerde planten en de controleplanten, en anderzijds tussen de twee behandelingen met mycorrhiza (Tabel 5.4 pg 71). De plantjes die groeiden op een substraat waarbij het inoculum was gehomogeniseerd met het substraat vertoonden een significant hogere planthoogte en wortelgewicht dan de andere behandelingen. Bij de controleplantjes waren deze groeikarakteristieken het laagst. Voor het scheutgewicht kon alleen een significante ($P \leq 0.05$) verhoging vastgesteld worden voor de behandeling waarbij het inoculum was gehomogeniseerd met het substraat. De wortelkolonisatie door de fungus gaf echter andere resultaten. Hier was de kolonisatie significant ($P \leq 0.01$) hoger waar het inoculum was toegediend in een laagje aan het substraat (Tabel 5.5 pg 72).

De hogere groeikarakteristieken van de plantjes waar de mycorrhiza waren gehomogeniseerd met het substraat waren waarschijnlijk het gevolg van een meer verspreid inoculum in het substraat. Wanneer de sporen gekiemd waren en de hyfen hun weg zochten naar de wortels om er te penetreren was de kans het grootst in de

behandeling waar het inoculum was gehomogeniseerd met het substraat, om in een korte tijd op een wortel te stoten om hiermee een symbiose aan te gaan. Dat kan als gevolg hebben gehad dat de symbiose in de behandeling waar de mycorrhiza gehomogeniseerd waren met het substraat, vroeger tot stand kon komen. De eerste weken na het tot stand komen van de symbiose transloceert de plant ongeveer 10 % van de C bestemd voor de wortels naar de fungus. Deze C is dus niet meer beschikbaar voor de plant. Hierdoor ontwikkelt de plant een goeiachterstand ten opzichte van niet-gemycorrhizeerde planten (zie experiment 1). Na enkele weken wordt deze groeiachterstand gecompenseerd door een groeiverbetering ten gevolge van de symbiose en ontwikkelt de gemycorrhizeerde plant een voorsprong op niet-gemycorrhizeerde planten. Het is dus aan te nemen dat de plantjes die groeiden op een substraat waarbij het inoculum gehomogeniseerd werd met het substraat reeds hun groeiachterstand hadden gecompenseerd en reeds sinds een bepaalde tijd een groeivoorsprong konden ontwikkelen. De plantjes die groeiden op een substraat waaraan het inoculum was toegevoegd in een laagje, bleken pas later van het groeivoordeel ten gevolge van de symbiose geprofiteerd te kunnen hebben. Het was duidelijk dat de plantjes van beide behandelingen reeds het stadium van groeireductie hadden verlaten aangezien de groeikarakteristieken meestal significant hoger waren in vergelijking met de controleplantjes. Hoe het verschil in groeirespons op de symbiose tussen de twee behandelingen zou zijn na een langer tijdsinterval is moeilijk te voorspellen.

Een significant hogere wortelkolonisatie kon worden waargenomen bij de behandeling waar het inoculum was toegevoegd in een laagje. Experimenten hebben echter aangewezen dat de frequentie van de wortelkolonizatie niet altijd in verband staat met de effectiviteit van de symbiose (Carroll *et al.*, 1985; Smith *et al.*, 1992). De vorming van arbuscules, de uitwisselingsplaatsen voor nutriënten tussen de fungus en de gastplant, is de meest belangrijke stap in de symbiose. Ook de 'branched absorbing structures (BAS)' (Bago *et al.*, 1998), vertakkingen van het mycelium buiten de wortel, moeten in beschouwing genomen worden, aangezien zij de plaats zijn waarlangs de mycorrhiza nutriënten opnemen uit de bodem. We kunnen dus enkel veronderstellen dat in de behandeling waar de mycorrhiza gehomogeniseerd werden met het substraat meer arbuscules en/of BAS gevormd werden en er dus een grotere uitwisseling van nutriënten kon plaatsvinden, resulterend in hogere groeikarakteristieken, vergeleken met de behandeling waar het inoculum was aangebracht in een laagje in het substraat.

Experiment 3: Interactie tussen mycorrhiza (*Glomus proliferum* en *Glomus* sp.) en *Radopholus similis* bij de *Musa* cultivar Poyo

In dit experiment was het de bedoeling de initiële aantrekking en penetratie van de nematode *R. similis* te onderzoeken in jonge bananenplantjes en tevens het effect van de aanwezigheid van AM-fungi op de nematoden na te gaan. De duur van het experiment was enkel 24 of 48 uren. Het experiment werd uitgevoerd in potcultuur.
In het eerste deel van het experiment werden de plantjes geïnoculeerd met *G. proliferum*, en 10 weken later werden nematoden van twee verschillende *R. similis* populaties (Australië en Cameroen) nabij de wortels geïnoculeerd (200 nematoden per plant). Na 24 uur werd de penetratie van de nematoden in de wortels nagegaan en hierbij werd ook de wortelkolonisatie door de fungus bepaald. Bijna geen nematoden penetreerden de wortel 24 uur na inoculatie (Tabel 5.6 pg 77) en de kolonisatie van de fungus 10 weken na inoculatie was 0 % (Tabel 5.7 pg 78). De fungus *G. proliferum* was nochtans een species dat voor het eerst gevonden werd op bananenplanten in Guadeloupe (Declerck *et al.*, 2000), dus dit species zou wortels van bananenplanten moeten kunnen koloniseren. Waarschijnlijk waren de omgevingscondities die aanwezig waren tijdens het experiment niet geschikt voor het ontwikkelen van dit species. De reden waarom 24 uur na inoculatie nog bijna geen nematoden de wortels gepenetreerd hadden kan een combinatie zijn van verschillende factoren: de tijd tussen incoluatie en observatie was te kort; het volume van de pot (250 ml) was te groot en de hoeveelheid geïnoculeerde nematoden (200 nematoden) was te laag. Met deze proefopzet was het bijna onmogelijk voor de nematoden om de wortels te bereiken en te penetreren binnen de 24 uren.
Daarom werd de proefopzet lichtjes gewijzigd. De tijd tussen inoculatie en observatie werd verlengd tot 48 uur en 400 nematoden werden geïnoculeerd. Er werd ook gebruik gemaakt van een andere fungus, namelijk *Glomus* sp. Wat betreft de penetratie van de nematoden in de wortel was er nog steeds het probleem dat er weinig nematoden konden teruggevonden worden na 48 uur (Tabel 5.6 pg 77). Het percentage van de wortels gekolonizeerd door de fungus was ongeveer 35 % (Tabel 5.7 pg 78). Sommige onderzoekers beweren dat mycorrhiza enkel effectief kunnen zijn als minstens 20 tot 30 % van de wortels gekolonizeerd is (Sanders *et al.*, 1977; Snellgrove *et al.*, 1982). Verder onderzoek heeft echter uitgewezen dat het percentage van infectie dat nodig is om effectief te zijn voornamelijk afhangt van het mycorrhiza species, de gastplant en de omgevingscondities. Saleh & Sikora (1984) vonden pas een positief effect van de mycorrhiza bij een kolonisatie van 55 %, terwijl Cooper & Grandison (1986) reeds een positief effect vaststelden bij een kolonisatie van 10 %.

Een bijkomende moeilijkheid in het vergelijken van wortelkolonisatie door de fungus is het feit dat verschillende species verschillende structuren vormen. In een aanvullend experiment in deze studie werd de kolonisatie van de wortel door *Glomus intraradices* bepaald om deze te vergelijken met de kolonisatie door *Glomus* sp. in dezelfde omgevingscondities. Het species *Glomus* sp. ontwikkelde een netwerk van hyfen waarbij bijna geen vesikels konden teruggevonden worden (Figuur 5.1 pg 79), terwijl het species *G. intraradices* een zeer dun netwerk van hyfen ontwikkelde en er veel vesikels konden waargenomen worden (Figuur 5.2 pg 80). Dit verschil in wortelkolonisatie vermoeilijkt het vergelijken van de kolonisatie door verschillende species.
Aangezien de nematodepenetratie in dit experiment behoorlijk laag was is het moeilijk enige conclusies te formuleren over het al dan niet voordelig zijn van de aanwezigheid van mycorrhiza.
Het is dus duidelijk dat deze proefopzet niet volledig geschikt was voor de studie van de penetratie van de nematoden, vlak na de inoculatie in de buurt van de wortels. Een langer tijdsinterval tussen inoculatie en observatie is daarom noodzakelijk. Om deze penetratie van dichterbij te kunnen bekijken kan ook gebruik gemaakt worden van een andere proefopzet (zie Deel II).

Deel II: Experimenten uitgevoerd in Petriplaten

Experiment 4: Aantrekking en penetratie van verschillende *Radopholus similis* populaties aan/in bananenplantjes

Om de aantrekking van de nematoden en de penetratie ervan vlak na inoculatie op de wortels te bestuderen, werden gecontroleerde condities ontwikkeld. Een eerste reeks van experimenten werden uitgevoerd in Petriplaten (∅ 5.5 cm), gevuld met zand. Hierin kon de baan die de nematoden afleggen alvorens in de wortel te penetreren, van dichtbij bestudeerd worden. Het experiment werd gestart met niet-gemycorrhizeerde wortels, om de meest geschikte condities uit te werken, zoals de hoeveelheid nematoden die geïnoculeerd moeten worden, de tijd tussen inoculatie en observatie, de vochtigheid van het zand, enzovoort. Het was de bedoeling om deze proefopzet dan later te gebruiken met gemycorrhizeerde wortels om de invloed van de aanwezigheid van AM-fungi op de penetratie van de nematoden na te gaan. De proefopzet bleek echter niet geschikt. Er werd gebruik gemaakt van een wortelsegment van 4.5 cm, wat betekent dat een stukje

van het wortelsysteem moest afgesneden worden. Dit veranderde de fysiologische toestand van de wortel. Ook bleek dat de nematoden een aantrekking hadden voor de plaats waar het wortelsegment van het wortelsysteem was afgesneden. De nematoden hadden de neiging op die bepaalde plaats te penetreren (Tabel 5.10 pg 84). Hieruit kon dus afgeleid worden dat ook deze proefopzet niet geschikt was om de penetratie van de nematoden, vlak na de inoculatie, te bestuderen.

Experiment 5: Initiële aantrekking van verschillende *Radopholus similis* populaties tot verschillende bananencultivars *in vitro*

In dit experiment werd de initiële aantrekking van nematoden tot de wortels van bananenplantjes bestudeerd in *in vitro* condities. Hiervoor werd gebruik gemaakt van twee verschillende nematodepopulaties, de sterk pathogene populatie afkomstig uit Oeganda, en de weinig pathogene populatie, afkomstig uit Indonesië. De twee verschillende bananencultivars die gebruikt werden waren Yangambi km 5, die gedeeltelijk resistent is tegen *R. similis*, en de cultivar Obino l'Ewai, die vatbaar is voor nematoden. De proefopzet bestond uit Petriplaten (∅ 13.5 cm) gevuld met MSR (Modified Strullu Romand) medium waarop een plantje was aangebracht. Dit geheel werd voor 24 uur in een donkere box bewaard zodat de wortelexudaten de mogelijkheid hadden zich te verspreiden in het medium. Daarna werden 5 nematoden per plaat op het medium gezet, zo ver mogelijk verwijderd van de wortels. De baan die de nematoden afgelegd hadden, enkele uren na de inoculatie, kon door een microscoop of zelfs met het blote oog geobserveerd worden.

Er werden geen verschillen waargenomen wat betreft de aantrekking van de nematoden naar de wortels van de plant, noch tussen de twee nematodenpopulaties, noch tussen de twee bananencultivars (Tabel 5.11 pg 88). Er waren ook geen verschillen zichtbaar tussen de verschillende behandelingen betreffende de baan die de nematoden afgelegd hadden alvorens in de wortel te penetreren.

De mechanismen die aanwezig zijn in een cultivar die resistentie vertoont tegen een bepaalde parasiet zijn variabel van cultivar tot cultivar. Voor Yangambi km 5 bestaan deze mechanismen onder andere uit een opbouw van fenolen en een accumulatie van flavonoiden en phytoalexines in het wortelstelsel na infectie door de nematoden. Deze mechanismen zijn post-infectioneel, dus zullen geen invloed uitoefenen op de aantrekking van de nematoden naar de plant. Ook de aanwezigheid van lignificatie in meerdere cellen van het wortelstelsel is pas voelbaar voor de nematoden na penetratie in

de wortel. Daarbij komt nog dat de gedeeltelijke resistentie van de cultivar Yangambi km 5, die reeds meerdere malen aangetoond werd *in vivo* (Wehunt *et al.*, 1978; Sarah *et al.*, 1992; Price *et al.*, 1994; Fogain & Gowen, 1997) nog niet bevestigd kon worden in *in vitro* condities (Stoffelen, 2000; Oroyaj, 2001). Er kan dus aangenomen worden dat in de vroege vegetatieve groeifase nog niet al de typen van resistentie tot ontwikkeling konden komen. Hierin kan dus een verklaring gevonden worden waarom er geen verschil in aantrekking van de nematoden kon waargenomen worden tussen de verschillende cultivars.

Het was ook onmogelijk enige verschillen waar te nemen in de aantrekking naar het wortelstelsel tussen de verschillende nematodenpopulaties met een verschil in pathogeniciteit. We kunnen daarom aannemen dat de stap van aantrekking naar de plant geen rol speelt in de pathogeniciteit van een populatie.

Volgens Bos & Parlevliet (1995) is de pathogeniciteit van een populatie afhankelijk van twee parameters, namelijk de virulentie (intensiteit van de symptomen) en de aggressiviteit (graad van aanval of infectie), waarbij de aggressiviteit op haar beurt afhangt van de graad van reproduktie en de mogelijkheid tot het penetreren van de wortels (Figuur 5.6 pg 91). Fallas & Sarah (1995) konden aantonen dat er een sterke correlatie bestaat tussen pathogeniciteit en de graad van reproduktie binnen een bepaalde populatie (zie grafiek, Annex 6). Hierdoor kan aangenomen worden dat de virulentie en de mogelijkheid tot penetratie of infectie geen invloed uitoefenen op het systeem of dat hun invloed samen een nul-effect geeft. Aangezien in de studie van Fallas & Sarah (1995) acht nematodepopulaties bestudeerd werden en een sterke correlatie aanwezig was in al de populaties, kan er aangenomen worden dat de virulentie en de mogelijkheid tot penetratie of infectie geen effect hebben op de pathogeniciteit van een populatie.

Besluit

In het eerste experiment over de invloed van AM-fungi op de penetratie en reproduktie van verschillende *Radopholus similis* populaties, werd een posifief effect van de aanwezigheid van AM-fungi waargenomen. Er werden significant minder nematoden teruggevonden in de gemycorrhizeerde wortels. Dit effect was meer uitgesproken bij de populatie afkomstig uit Ivoorkust. Er konden echter geen verschillen waargenomen worden in de groeikarakteristieken van de gastplant. Ook kon het verschil in pathogeniciteit tussen de twee populaties (Ivoorkust en Australië) bevestigd worden. De nematoden bleken geen effect te hebben op de wortelkolonisatie door de fungus wanneer

de fungus 4 weken vooralleer de wortels in contact kwamen met de nematoden werd geïnoculeerd.
In een bijkomend experiment werden twee methoden voor het inoculeren van de fungus vergeleken. Er werd een betere groeirespons vastgesteld in de behandeling waarbij het inoculum werd gehomogeniseerd met het substraat, 9 weken na de inoculatie met de fungus. Er werd daarentegen een hogere wortelkolonisatie door de fungus vastgesteld in de behandeling waarbij het inoculum in een laagje was aangebracht, omgeven door twee lagen van substraat. Er kan aangenomen worden dat de ontwikkeling van arbuscules, de plaats waar de uitwisseling van nutriënten gebeurt tussen de fungus en de gastplant en/of de ontwikkeling van 'branched absorbing structures (BAS)', een vertakking van het mycelium in de bodem, dus de plaats waar de mycorrhiza nutrienten opnemen uit de bodem, meer uitgesproken was in de behandeling waarbij het inoculum was gehomogeniseerd met het substraat. Dit kan dus geleid hebben tot een betere groeirespons. Om dit te kunnen bevestigen is het noodzakelijk de vorming van arbuscules in de wortelsystemen van de twee behandelingen verder te onderzoeken en indien mogelijk, de vorming van BAS in het substraat. Er konden geen voorspellingen gemaakt worden over de groeikarakteristieken van de planten in de verschillende behandelingen voor een langere tijdsschaal. Het was dan ook onmogelijk één van de twee inoculatiemethoden te verkiezen boven de andere.
De proefopzet in potcultuur is geschikt voor het bestuderen van de reproduktie van nematoden in de wortels. In een derde experiment werd deze proefopzet echter gebruikt voor het bestuderen van de mechanismen die tussenkomen in de aantrekking van de nematoden naar de wortel en de penetratie van de nematoden in de wortel vlak na de inoculatie. De bekomen resultaten gaven aan dat de tijd tussen inoculatie en observatie niet voldoende was om voldoende penetratie van de nematoden in de wortels te verkrijgen.

Daarvoor werd gezocht naar een proefopzet in meer gecontroleerde condities, namelijk in Petriplaten. In een eerste experiment, uitgevoerd in Petriplaten gevuld met zand, werd enkel een wortelsegment geinoculeerd met nematoden om de penetratie van de nematoden in de wortel van dichtbij te kunnen bestuderen. De schade die aan de wortel werd aangebracht wanneer het wortelsegment van het wortelsysteem werd afgesneden wijzigde echter de fysiologische status van de wortel, waardoor misleidende resultaten werden bekomen. De nematoden hadden de neiging de wortel te penetreren op de plaats waar de wortel beschadigd was, zelfs al werden ze aan de tegenovergestelde kant, aan het meristeem, geinoculeerd. Een oplossing voor dit probleem werd gevonden in een nieuwe

proefopzet. Hier werden grotere Petriplaten gebruikt zodat het plantje in zijn geheel in de plaat kon gebracht worden. Om de aantrekking van de nematoden naar de wortel te bestuderen werden twee verschillende nematodenpopulaties en twee verschillende bananencultivars gebruikt. Er konden geen verschillen waargenomen worden in de aantrekking van de nematoden naar de wortels in de verschillende behandelingen. Dit is waarschijnlijk te wijten aan het feit dat verschillende nematodenpopulaties met een verschil in pathogeniciteit toch een gelijke mogelijkheid tot penetreren van de wortel vertonen, en dat de meeste van de resistentiemechanismen van de gastplant post-infectioneel zijn, dus slechts tot uiting komen wanneer de nematoden reeds gepenetreerd zijn in de wortel. Meer nog, de resistentie van Yangambi km 5, een cultivar die gedeeltelijk resistent is *in vivo*, bleek nog niet bevestigd te zijn *in vitro*.

Summary

World banana production is threatened by various diseases and pests, which include migratory endoparasitic nematodes. In commercial plantations, these plant-parasitic nematodes are controlled by the use of nematicides but the high environmental toxicity and especially the high price of these products makes them not suitable for the use in small-scale production. Therefore other control means, like the use of biological antagonists, are under study. Endomycorrhizal fungi are known to play an important role in the biological control of plant-parasitic nematodes.

In the first experiment, a positive influence of the presence of arbuscular mycorrhizal fungi (AM-fungi) could be observed on the reproduction of different *Radopholus similis* populations in the roots of banana plantlets. No differences in plant growth characteristics could be detected, but less nematodes were recovered from the mycorrhized roots compared to the non-mycorrhized roots. Also the higher pathogenicity of the population from Ivory Coast compared to the population from Australia could be confirmed.

Two different procedures of inoculation of the AM-fungus were tested in a second experiment. When the inoculum was added homogeneously to the substrate, the highest plant growth characteristics were obtained, while when the inoculum was added in a layer, between two layers of substrate, the highest root colonization by the AM-fungus could be obtained.

In a next experiment, an experimental setup in culture chamber was conducted to study only the attraction and penetration of nematodes directly after inoculation. As almost no nematodes could be recovered from the roots, the time interval between inoculation and observation had to be questioned.

Petri dish experiments were conducted to obtain more controlled conditions to study the attraction and penetration of nematodes to/into the roots. In a first Petri dish experiment, an excised root segment embedded in sand was inoculated with nematodes to obtain a closer view on the behavior of the nematodes. The physical damage due to the excision of the root changed the physiological status of the root segment, giving deceiving results. Therefore, larger Petri dishes were used so the whole plant could be placed in the dish. The attraction of different *R. similis* populations to different banana cultivars, directly after inoculation could be studied. No differences could be observed in the attraction between the different treatments.

Contents

List of abbreviations

AM-fungi	Arbuscular Mycorrhizal fungi
AMIS	Advanced Methods for Innovation in Science
ANOVA	Analysis of Variance
BAS	Branched Absorbing Structures
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
FAO	Food and agriculture organization
INIBAP	International Network for the Improvement of Banana and Plantain
IPM	Integrated Pest Management
MS	Murashige & Skoog
MSR	Modified Strullu Romand
Ri T-DNA	*Agrobacterium rhizogenes* Riker Transformed Deoxyribonucleid acid
RMD	Relative Mycorrhizal Dependency

1 Introduction

Banana and plantains (*Musa* spp.) are staple foods for 400 million people throughout the tropics, and for another 600 million people they give 25 % of the basic nutritional needs. As only 15 % of the world production enters world trade, the banana plants are mainly grown by smallholders for local consumption. There are a lot of pests and diseases that threaten the banana production reducing the yield. In most regions of the world, plant-parasitic 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. Because of the weakened root system, plants can topple when bearing fruits or under strong windstorms. This results in annual yield losses of about 20 % of the worldwide production.

Nematode management is usually based on chemical control. Nevertheless the negative aspects of chemical nematicides such as their price and their high environmental toxicity result in the search for other control means like the use of biological antagonists.

During the last two decades, many studies have been carried out on the use of arbuscular mycorrhizal fungi (AM-fungi) as a possible biological control agent. A review of the literature indicated that the majority of host/fungus/nematode interactions resulted in increased host plant tolerance or resistance to nematodes in mycorrhized plants compared to non-mycorrhized plants. This is probably due to the increased nutrient uptake in the presence of mycorrhiza, resulting in more vigorous plants or a change in the physiological status of the host. Assumptions about other mechanisms, involved in the reduction of the nematode populations in mycorrhized roots were done, but the exact mechanisms remain uncertain. Factors such as microbial changes in the rhizosphere, competition for host photosynthates and changes in the root morphology are thought to play a role in the beneficial effect of AM-fungi against plant-parasitic nematodes.

The symbiosis between AM-fungi and banana is non-host specific. A single plant species can associate with most (if not all) known AM-fungi, while a single fungal isolate can infect a vast array of host species. Experiments however have shown that the efficiency of the symbiosis can differ between different fungus/host pairs. The influence of a mycorrhizal strain as a control agent against plant-parasitic nematodes can also differ between different nematode populations. This results in unique host/fungus/nematode interactions, making this subject one of serious complexity. The experiments performed in this work are part of this complex unity.

In a first set of experiments, different mycorrhizal strains and different nematode populations were used to investigate the interaction between mycorrhiza and nematodes on banana plants. These experiments were performed in semi-controlled conditions (climatic chamber). In a further group of experiments, a new experimental setup was developed to have more controlled conditions (Petri dish). The aim of these experiments was to have a more intimate approach on the mechanisms involved in the attraction and penetration of nematodes to/into banana roots.

2 Overview of the literature

2.1 The banana

2.1.1 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 Scitaminae and is composed of only two genera: *Musa* and *Ensete* (Simmonds, 1966). The genus *Musa* contains 50 species classified into 5 sections: *Rhodochlamys, Callimusa, Ingentimusa, Eumusa* and *Australimusa* (Jones, 2000), 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 genome) and *Musa balbisiana* Colla (BB genome), originating from Asia, are the parents of the agricultural produced banana of this section (Gowen, 1988). Over time mutation led to the evolution of parthenocarpy (i.e. the ability to produce fruit without fertilization) and female infertility, resulting in the production of edible and seedless fruit. Furthermore, the absence of meiosis in the female gametes led to the formation of triploids and tetraploids (Stover & Simmonds, 1987). Most of the cultivated varieties however are triploid (3n=33) including the commercial Cavendish AAA varieties.

Another important step in the evolution of the banana was the natural crossing that took place between *M. balbisiana* and AA diploids to produce AB diploids and AAB, ABB triploid hybrids. This extended the range of plant characteristics and led to the adaptation to other conditions such as drought tolerance, which allowed the spread of cultivation from the lowland humid tropics to seasonally drier zones (INIBAP, 1993). Currently, the most important genome types are AAA, AAB and ABB.

2.1.2 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.

The corm is the real stem of the plant and is situated right under the ground level. It is an important storage organ. The size of the corm is related to the size of the plant, itself a function of the age and growing conditions of the variety. A diameter of 15 to 20 cm can

be considered as a mean diameter of a corm. Internally the corm is distinguished into two main regions, a central cylinder consisting of a starchy parenchyma surrounded by a cortex of 1 to 3 cm thick. The outer layer of the central cylinder is a cambium-like, meristematic tissue from where the roots develop and is called the ring of Mangin.

Banana plants may have as many as 300 to 500 primary roots. They are about 5 to 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 (Swennen & Rosales, 1994). Growth rates of the tips may reach 60 cm per month, or around 1.5 to 3 cm a day (Lassoudière, 1978). From the primary roots, a system of secondary and tertiary roots and root hairs develops. Secondary roots may have a diameter from 0.5 to 3.5 mm while the diameter of tertiary roots is even less than 0.2 mm (Laville, 1964). The major part of the root system is situated in the upper part of the soil. The root distribution is thus limited to the upper 40 to 50 cm but is, however, strongly influenced by soil type and drainage.

The vegetative buds also arise from the corm. They form shoots that can evolve in new stems, called suckers. Five 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 a 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 2.1). 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 to 3.8 leaves per month and in winter, in the cool subtropics, it can fall to 0.1 to 1.2 leaves per month (Jones, 2000).

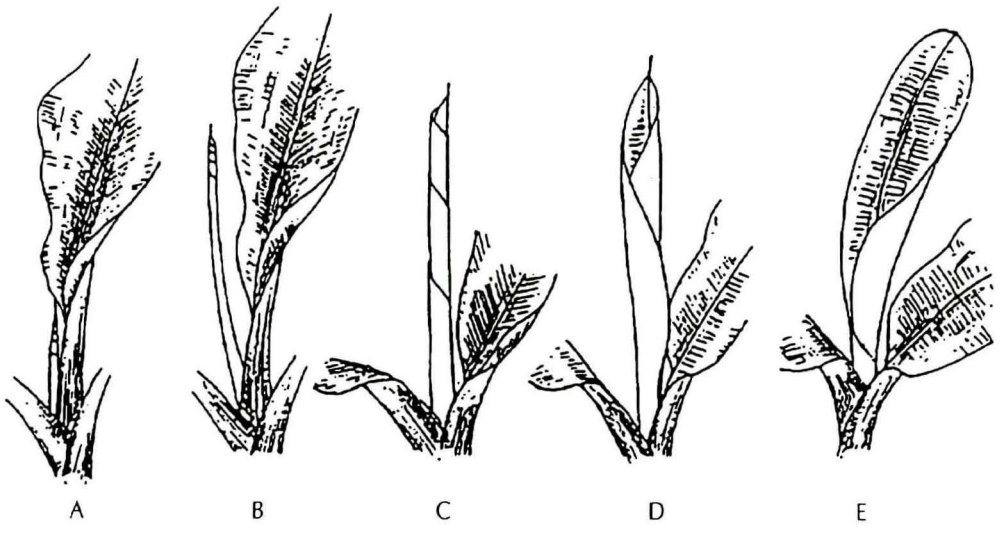


Figure 2.1: Stages of the unfurling of the heart leaf of a banana plant (after Brun, 1962).

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, which are tightly enclosed in bracts. 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 among 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 15 fingers (Samson, 1986) and may weigh up to 30 kg (Gowen, 1988) (or sometimes even more).

2.1.3 Cultivation

Bananas are cultivated over a wide variety of 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). The production of bananas requires 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 1 gives an overview of the diversity of soils that can be suitable and their main associated constraints apart from topography and climate (Delvaux, 1995).

As bananas are high demanding crops, the 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. Also P, Ca, Mg and sometimes S and Zn can have an importance. The rate of application depends on climate, soil type, variety, management practices and yield goal.
The drawback of high-input cropping systems is the rapid environmental degradation, so soil fertility research is clearly needed to meet the demand for sustainable agricultural production (Scholes *et al.*, 1994).

Bananas may grow as a permanent crop or on a system of replanting every 3 to 8 years (Stover & Simmonds, 1987). In many countries, especially in the Caribbean, Surinam, Ivory Coast, Cameroon and the Pacific, bananas and plantains soon become unproductive for reasons related to the soil structure, fertility, drainage and severity of pathogens. Consequently, frequent re-plantings are necessary.

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

- The industrial production of bananas (mostly for export markets). The cultivation of these bananas, mainly dessert bananas, is often oriented towards very large plantations, long plantation life, intensive 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. 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. The bananas are often intercropped with maize and beans in mixed farming systems, providing a source of food and income throughout the year.

2.1.4 Economic importance

The banana is cultivated in more than 120 countries in the tropical and subtropical regions of the world, especially in the Latin American-Caribbean region, Africa and the Asian-Pacific region (Jones, 2000) (Figure 2.2). They are grown over a harvested area of approximately 10 million hectares with an annual production of around 96 million metric tons (FAO, 2001). They are the developing world's fourth most important food crop after rice, wheat and maize in terms of gross value of production (FAO, 2001).

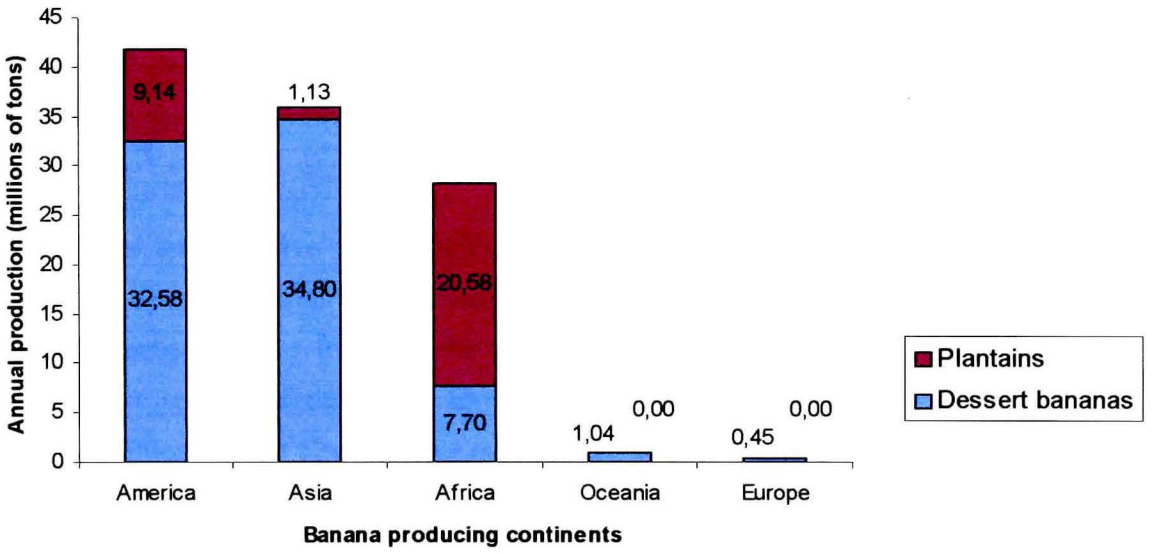


Figure 2.2: Annual production of dessert bananas and cooking bananas (FAO, 2001).

The vast majority of producers are small-scale farmers growing the crop either for home consumption or for local markets. Only about 15 % of the total banana production reaches the export market. The remaining 80 million tons are consumed locally, highlighting the importance of this food crop in many communities (Figure 2.3; FAO, 2001). Banana is the major staple food crop for 400 million people throughout the humid and the subhumid tropics and for another 600 million people it gives 25 % of the basic nutritional needs. While it is estimated that people in North America and Europe eat no more than 30 grams of banana per day (about one banana a week), citizens of some African countries consume up to 500 grams each day, providing as much as 25 % of their calorie intake (INIBAP, 1993).

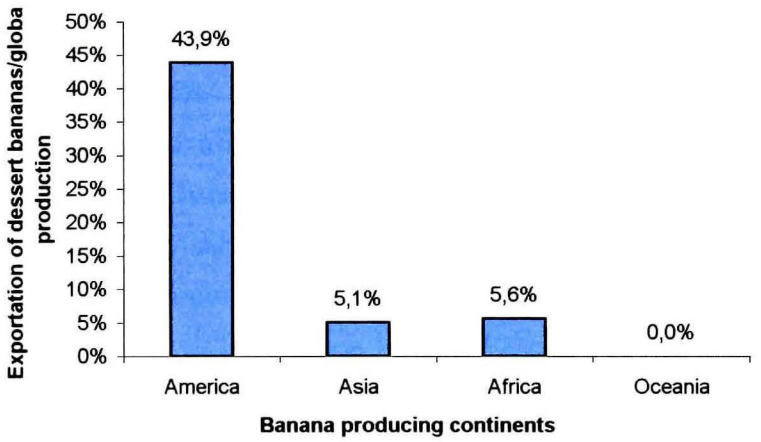


Figure 2.3: Percentage of dessert bananas that are exported in comparison with the global production for each continent (FAO, 2001). Data for Europe not available.

Banana is an attractive perennial crop for farmers in developing countries for many reasons. The banana is very cheap to produce. It can grow in a wide range of environments and the fruit can be produced all year round providing a steady cash income or supply of nutritious food. Bananas are a high-yielding source of dietary carbohydrates and are also rich in a number of vitamins (A, B1, B2, B6, C) and important minerals (potassium, phosphorus and calcium). Cooking bananas provide the same nutritional value, although the carbohydrate is present as starch rather than sugar.

2.1.5 Uses of *Musa*

The dessert bananas (AAA) are usually eaten raw and ripe as a fruit, whereas more starchy bananas (AAB, ABB) are consumed as a cooked vegetable, using boiling, steaming, roasting, baking and frying. Because they contain vitamin A, bananas aid the digestion, and it is reported that boiled, mashed ripe fruit can be good for constipation. The juice from the male bud provides an apparent remedy for stomach problems, while there are reports of the ripe fruit being used in the treatment of asthma and bronchitis (INIBAP, 2001). The juice from the ripe fruit of varieties known as "beer bananas" may be drunk fresh or fermented to make a beer with a low alcohol content and a short shelf-life. The beer is important nutritionally and is rich in vitamin B due to the yeast content. Also other parts of the banana plant can be eaten. The banana "heart" can be removed

from the pseudostem after harvest and can be cooked. It is like celery, with a texture and taste similar to bamboo shoots. New shoots and male buds can be cooked as a vegetable. Another species, *Musa textilis*, is frequently used as a source of fibre. It is used extensively in the manufacture of certain papers.

The large leaves of bananas are put to a diversity of uses, for example an umbrella, a "biological plate" for serving food, a hat, etc.

In general, it can be concluded that the banana plant possesses many major and minor properties (INIBAP, 2001), which highlight the great importance of this crop in many cultures.

2.1.6 Banana pests and diseases

Diseases are among the most significant constraints to banana production in the tropics and 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 spreading 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 the black and yellow Sigatoka, caused by the fungi *Mycosphaerella fijiensis* and *Mycosphaerella musicola*, respectively. These and other foliar diseases can cause serious damages during periods of high rainfall but can be controlled with protectant and systemic fungicides and plantation sanitation (Mourichon *et al.*, 1997).

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 the 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. In addition, the weakened root system affects plant anchorage resulting in plant toppling, especially at bunch filling and when strong winds prevail. Plant-parasitic nematodes are widespread and cause severe crop losses in commercial banana plantations. Five nematode species are considered the most important for bananas: the burrowing nematode *Radopholus similis*, the lesion nematodes *Pratylenchus coffeae* and *P. goodeyi*, the spiral nematode *Helicotylenchus multicinctus* and the root-knot nematode *Meloidogyne* spp. (Gowen & Quénéhervé, 1990). The burrowing and lesion nematodes are the most damaging pests on banana in the tropics, but the spiral nematode is more important in the subtropics (see paragraph 2.2.1.6).

2.2 Nematodes

2.2.1 Nematodes attacking several crops

2.2.1.1 Introduction

Nematodes constitute one of the most important and abundant group within the animal kingdom. Table 2.1 illustrates the position of the nematodes in the animal kingdom. 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 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).

Table 2.1: Position of Nematodes in Animal Kingdom (Hyman, 1940, 1951).

Protozoa
Metazoa
- Mesozoa
- Parazoa
- Eumetazoa
  - Radiata
    - Phylum Cnidaria (Coelenterata)
    - Ctenophora
  - Bilateria
    - Acoelomata
      - Platyhelminthes
      - Rhynchocoela (Nemertinea)
    - Pseudocoelomata
      - Acanthocephala
      - Aschelminthes
        - Class Rotifera
        - Gastrotricha
        - Kinorhyncha
        - Priapulida
        - **Nematoda**
        - Nematomorpha
      - Entoprocta
    - Coelomata

Plant-parasitic nematodes are the most damaging pests of banana and are one of the greatest threat to this crop throughout the world. Nematodes alone or in combination with other soil micro-organisms 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 80 billion US dollars worldwide each year (Barker *et al.*, 1994), and it is considered that all crops are damaged by at least one nematode species.

2.2.1.2 Morphology and anatomy

Besides their wide ecological diversity, the different nematode species are fairly alike in structure. Plant-parasitic nematodes are small, 300 to 1000 μm long (with some up to 4 mm), by 15 to 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 (Wallace, 1963). Nematodes have an elongate digestive and reproduction system inside a resistant multi-layered outer cuticle (usually transparent). For feeding, nematodes use a stylet: a hard, sharp spear. Figure 2.4 shows the anatomy of a plant-parasitic nematode.

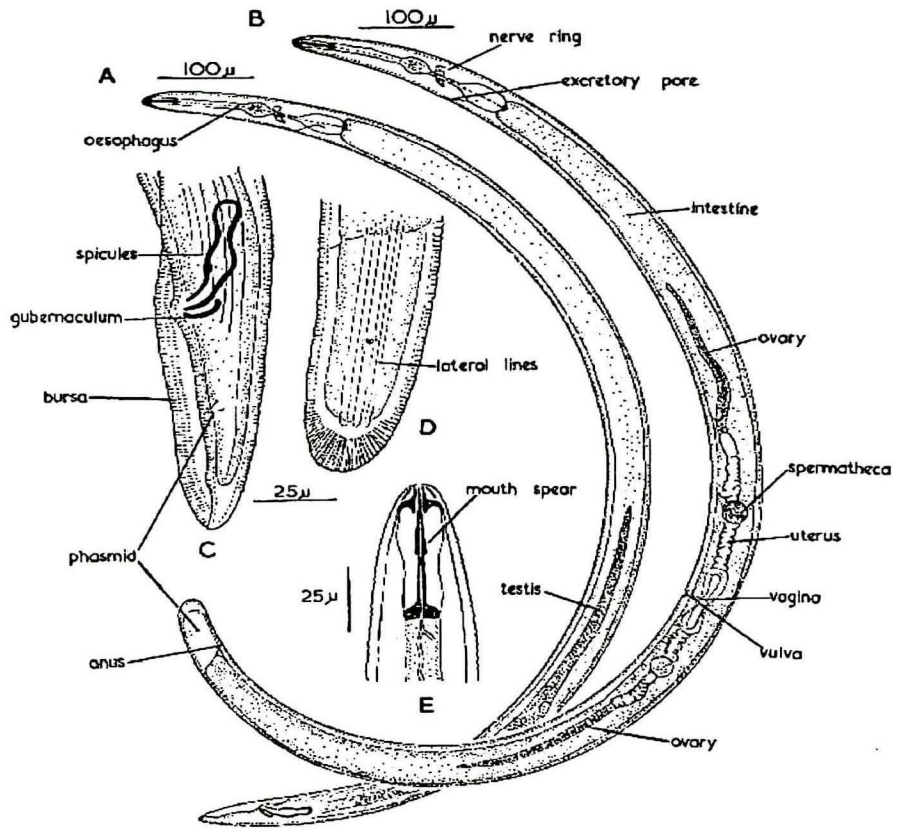


Figure 2.4: Detailed anatomy of a typical plant-parasitic nematode (Wallace, 1963).
(A) male ; (B) female ; (C) male tail ; (D) female tail ; (E) head.

2.2.1.3 Life cycle and reproduction

Plant-parasitic nematodes have a fairly simple life cycle which consists of six stages: the egg, four juveniles, 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 (Luc *et al.*, 1990).

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 for years but hatch quickly when a host plant grows near them.

2.2.1.4 Types of nematodes

In general, based on their biology, four types of plant-parasitic root nematodes can be distinguished (Sarah, pers. communic.):

Ectoparasitic nematodes always remain outside the plant. They are nearly all migratory i.e. they are mobile and can freely move from the outside of 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 layers of cells (Speijer & De Waele, 1997). Examples are sting nematodes (*Belonolaimus* spp.), stubby-root nematodes (*Trichodorus* spp.) and ring nematodes (*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 nematodes (*Rotylenchulus* spp.) and citrus nematodes (*Tylenchulus* spp.).

Migratory endoparasitic nematodes live completely inside the host plant but are able to move freely between the roots and the soil. In the roots, they remain migratory (mobile) and can feed on numerous different cells. 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 (Speijer & De Waele, 1997). Examples are spiral nematodes (*Helicotylenchus* spp.) in banana, lesion nematodes (*Pratylenchus* spp.) and the burrowing nematode (*Radopholus similis*).

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 swellings of the root that are commonly called gall or knot (Speijer & De Waele, 1997). Examples are root-knot nematodes (*Meloidodyne* spp.) and cyst nematodes (*Heterodera* spp.).

2.2.1.5 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 the pathogens can produce individually (Agrios, 1997).

2.2.1.6 Nematode species that cause serious damage to banana

Many nematode species attack roots of banana. They belong to almost 150 species and represent 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 or blackhead toppling disease (Sarah, 2000) (see paragraph 2.2.2).

Pratylenchus coffeae and ***P. goodeyi*** (the lesion nematodes) can attack the roots of all kinds of plants (Gowen, 1993). *Pratylenchus coffeae* is the most widespread species on cooking bananas in Central America and the Caribbean. *Pratylenchus 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 nematode invades the cortical parenchyma of the roots, but in contrast to the burrowing nematode and the lesion nematode which attack the entire banana root cortex, the spiral nematode colonizes only the superficial cortical layers (Orion *et al.*, 1999).

***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).

2.2.2 *Radopholus similis*

2.2.2.1 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 2.5). 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 (Sarah, 1989; Gowen & Quénéhervé, 1990; Stover & Simmonds, 1997; Sarah, 2000).

During the late 1950's to early 1970's, 'Gros Michel' banana plantations were replaced by the Fusarium wilt resistant Cavendish cultivars, which were more susceptible to the burrowing nematode. As a result, *R. 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).

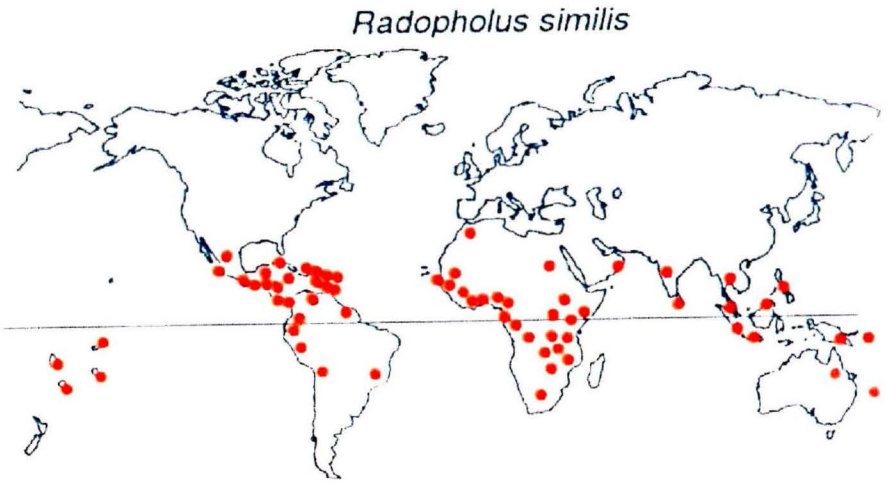


Figure 2.5: Worldwide distribution of *Radopholus similis* on bananas and plantains (after Bridge, 1993).

2.2.2.2 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 to 32 °C the life cycle takes 20 to 25 days; fertilization is usual but also parthenogenesis may occur (Orton & Siddiqi, 1973). Only the females (Figure 2.6) and juveniles penetrate and parasitize the host tissue; males (Figure 2.7) 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 to 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 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 to 6 eggs per female per day). Eggs hatch occurs in 8 to 10 days, and the four juvenile stages occur over the following 10 to 13 days.

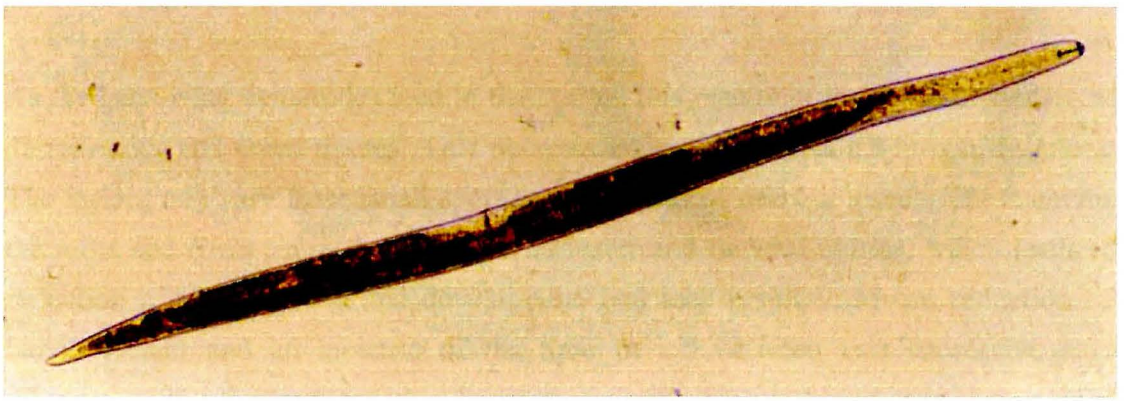


Figure 2.6: Picture of *Radopholus similis* (female) observed under a 100 x magnification.

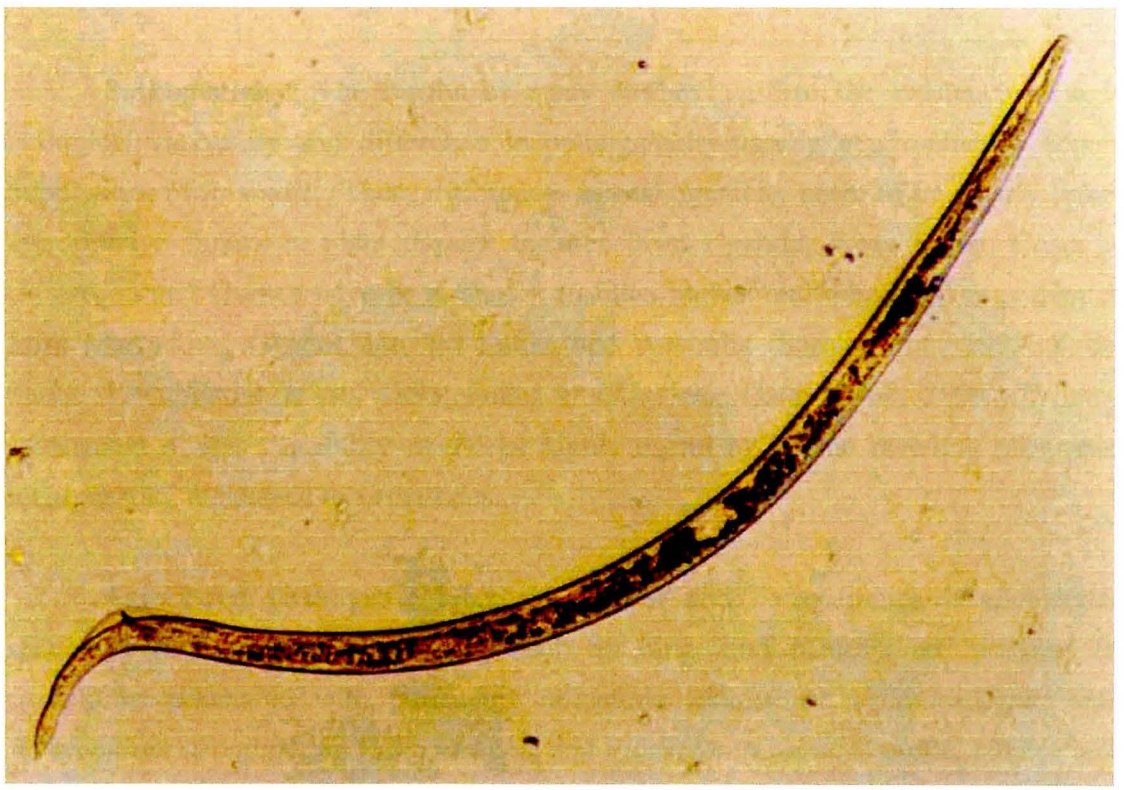


Figure 2.7: Picture of *Radopholus similis* (male) observed under a 100 x magnification.

2.2.2.3 Symptoms and pathogenicity

As the burrowing nematodes feed in the cortex, this 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 cause the reduction of water and mineral uptake, which leads to the reduction of plant growth and development and may result in 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 plants can fall because of their own weight and/or strong windstorms (toppling disease). Crop losses depend on several factors, including the pathogenicity of the local burrowing nematode population, associated pathogens (including other nematode species), banana cultivars, climatic conditions and soil factors (especially fertility) (Sarah, 2000).

Pathogenicity: The results of many studies confirm the existence of a 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 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 cultivars Poyo and Grand Naine of the Cavendish subgroup are highly susceptible, while the cultivars Yangambi km 5 and Pisang Jari Buaya have 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 infections are high in poor and eroded soils, but are much lower when the soil has a high fertility (Sarah, 1989; Sarah, 1995).

2.2.2.4 Damage and economic importance

Nematode damage is often a limiting factor in banana production, making *R. similis* a pest of substantial economic importance. Levels at which nematodes cause economic losses may vary between seasons of the year, different sites and populations (Gowen, 1979) and therefore must be evaluated on a local basis (O'Bannon, 1977).
Yield reduction of 30 to 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 % (Sarah, 1989). All over the world, banana companies spend millions of US dollars on nematicides and their application. Furthermore strong winds lead to yield losses due to uprooting or toppling of plants ranging from 104 to 156 bunches per ha per year, causing another loss of around 4 million US dollars (Davide, 1994).
In general, the annual global loss in agriculture due to damage by plant-parasitic nematodes has been estimated as 100 billion US dollars worldwide (Oka *et al.*, 2000).

2.2.3 Nematode Control

Several methods to effectively control 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 by the use of chemicals, such as various types of nematicides. Other control methods use cultural practices such as crop rotation, fallow and cover crops, physical agents such as heat and flooding or cultivars who show resistance or partially resistance against some species of plant-parasitic nematodes. Finally biological control with the use of antagonist bacteria and fungi is possible. In

practice, a combination of several methods is usually used for controlling nematode diseases of plants.

2.2.3.1 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 the international export market. However, nematicide use is slated for reduction due to potential environmental problems, human and animal health concerns, side effects to non-target organisms, contamination of surface and ground water, etc. For local market the use of these pesticides is not possible nor justified.

The widely used nematicides are generally non-volatile organophosphates or carbamates (Mink *et al.*, 1989). Their use is even expected to increase following the withdrawal of methyl bromide, that already has been banned in some countries and for which withdrawal from the market is planned for most countries by international agreements. The increase of organophosphates and carbamates however will bring about new environmental concerns. Other effective nematicides such as DBCP (dibromochloropropane) and EDB (ethylene dibromide) already have been withdrawn from the market due to their possible deleterious effects on humans and the environment (Oka *et al.*, 2000).

Most of the nematicides 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. To overcome this problem and to improve the efficacy of the treatment, several factors have to be considered, especially the biological factors governing nematode fluctuations and the environmental factors which affect nematicide activity. Quénéhervé *et al.* (1991), for example, obtained high reductions in nematodes population when chemicals were applied on an individual plant basis at the time of harvest to protect the following crop.

2.2.3.2 Cultural practices

With increasing awareness of hazards associated with chemical pesticides, research has been directed towards development of nematode management (instead of nematode control) systems that operate with reduced nematicide inputs and increased cultural control practices in an integrated pest management (IPM) (Kaskaija *et al.*, 1999). This concept is defined as "the coordinated use of all possible control methods for pests, including biological, environmental, and cultural methods, within management techniques directed towards the fullest utilization of natural pest mortality and other suppressive factors in any given agrosystem" (Chiarappa *et al.*, 1972). It is this concept that agricultural research needs to continue developing, as it would yield control measures applicable to both the intensive banana agrosystem and the resource-poor farmers' banana-based agrosystem. Different nematode IPM control techniques currently in use are crop rotation, intercropping, hot water treatment, soil amendments, resistant cultivars, etc. (Kashaija *et al.*, 1999).

In the tropics and subtropics, crops are often grown continuously. These methods of monocultivation lead to an increase in all pests present in the soil. Monocultural succession can be interrupted by means of fallow. *Radopholus similis* can not be totally eradicated this way because it can survive long periods in decaying roots or pieces of rhizome. It can even survive for 9 to 18 months in the total absence of a host plant (Loos, 1961). The decreased level of nematode infestation however may allow a reduction in the number of nematicide applications. There are some serious drawbacks about fallowing. Firstly, the site needs regular tillage or herbicide application to prevent, or at least limit, the development of weeds which might be hosts of nematodes. Secondly, if the soil is left for a long period without a cover crop, erosion risks which are serious in tropical countries are increased. Finally, a long absence of a cover crop might cause a serious decrease in fertility (Sarah, 1989).
Because of these disadvantages, an alternative to clean fallow is the use of non-host cover crops to maintain or even improve the soil fertility. The cover crops can also be used as a food crop, giving another advantage above clean fallow. In different experiments, sorghum, hairy indigo, cowpea, tomato, sugarcane and pepper have all reduced nematode populations (McSorley & Dickson, 1995; McSorley *et al.*, 1999). 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 infection (Sarah, 1995). Soil ploughing before planting allows the root system to develop better 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, including natural enemies of nematodes (Rodriguez-Kabana *et al.*, 1987).
Organic soil amendments which reduce nematode populations generally have high nitrogen contents relative to carbon. Oil cakes, animal manure and chitinaceous and proteinaceous compounds are such amendments. Ammonia released from organic amendments during microbial decomposition plays an important role in nematode control (Oka *et al.*, 2000).

2.2.3.3 Physical control

Soil treatment is insufficient to avoid a rapid contamination of roots and rhizomes, because planting material is generally the principal source of infestation. It is therefore recommended that soil treatments be complemented with the use of disinfected or nematode-free planting material.
The simplest disinfecting technique consists of peeling the corms to eliminate nematode lesions. However, this is not sufficient since nematodes may be located deep within the cortex and produce no visible symptoms. De Jager & Rabie (1991) reported that 33 % of the nematodes penetrated deeper than the epidermis which is usually removed during peeling rendering it an ineffective method. Peeling in combination with coating the planting material with a nematicide mud gave promising results. In some production areas, hot-water treatments are implemented, but this technique is labor-intensive and requires careful monitoring since temperature and immersion time are critical.
Nematode-free planting material can be obtained easily by using plants propagated *in vitro*. 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.

In 1976 and 1982, exceptionally large rainfall caused flooding of hundreds of hectares in the Nieky Valley in the Ivory Coast. This natural disaster caused a sharp decrease in nematode populations resulting in an increase in banana production in the zones where

the flooding had lasted for 5 to 7 weeks. The dramatic reduction in nematode numbers was probably 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 technique used to reduce nematode populations in the soil is soil solarization. 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.

2.2.3.4 Control by resistant cultivars

Growing nematode resistant or tolerant cultivars is considered as an effective and sustainable method for nematode management. It is probably the best form of nematode control, especially for resource-poor farmers who cannot afford the high cost of nematicides (Kashaija *et al.*, 1999).
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/susceptibility on the one hand and tolerance/sensitivity on the other hand are defined as independent, relative qualities of a host plant based on comparisons between cultivars. A host plant may either suppress (resistance) or allow (susceptibility) nematode development and reproduction; it may suffer either little injury (tolerance), even when heavily infected with nematodes, or much injury (sensitivity), even when relatively lightly 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 cultivars 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 (*Musa* AAA), Kunnan (*Musa* AB) (Collingborn & Gowen, 1998; Collingborn *et al.*, 2000) and two Pisang Jari Buaya (*Musa* AA) accessions of the

Eumusa bananas (Wehunt *et al.*, 1978; Sarah *et al.*, 1992; Price, 1994; Fogain & Gowen, 1997), and the 'Rimina' and 'Menei' accessions of the *Australimusa* group (Stoffelen, 2000; Stoffelen *et al.*, 2000).

At present, the mechanisms of resistance are not clearly understood, but it is thought that cell wall thickenings along with phenolic acid complexes are possible mechanisms of resistance (Fogain & Gowen, 1996). Valette *et al.* (1997) also found post-infectional production of phenols in the roots of Yangambi km 5, but not in the susceptible Poyo. Valette *et al.* (1996) detected a more extended accumulation of flavonoids, dopamine, caffeic esters and lignin in the roots of an infected resistant cultivar as compared to an infected susceptible cultivar. This may also contribute to the plant defense strategy, limiting nematode ingress within the roots.
The production of *de novo* defense compounds (phytoalexines) by plants in response to biotic, physical or chemical agents is well documented. Binks *et al.* (1997) showed a significant increase in phytoalexin production in root samples from plants which had been inoculated with nematodes, compared to control plants. It can thus be suggested that this compound could be playing a significant role in the plant defense system. Collingborn *et al.* (2000) found consistently higher levels of condensed tannins in a resistant cultivar, compared to two susceptible cultivars, indicating that they also could be involved in the resistance mechanism.

A better knowledge of the 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 it possible to transfer genes from microorganisms, plants or animals to higher plants, so engineered nematode tolerant or resistant cultivars are now within reach.

2.2.3.5 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 fungus *Paecilomyces lilacinus*, which is a parasite of burrowing nematode eggs, juveniles and adults, has been found to reduce *R. similis* populations (Davide, 1994). It is also believed that antagonistic fungi like *Arthrobotrys* spp. and *Paecilomyces pilacinus* and the rhizobacteria (*Pseudomonas* spp.) can be potential control agents of nematodes (Kashaija *et al.*, 1999). For example, the bacteria *Pseudomonas fluorescens* and *P. putida* have been shown to inhibit the invasion of roots of 'Grand Nain' (AAA) (Aalten *et al.*, 1998).
Also nematodes can be used as biological control agents. The entomopathogenic nematodes *Steinernema carpocapsae* and *S. glaseri* were found to suppress the penetration of *Meloidogyne javanica* on tomato roots. These nematodes are attracted to tomato roots so the suppressive effect is probably due to competition for space (Ishibashi & Choi, 1991).

Recent attempts have been successful in reducing the nematode populations by inoculation with mycorrhizal fungi. Several studies on the interactions between different mycorrhizal strains and different nematode populations have been undertaken, and revealed that various interactions exist. Nevertheless, in most cases mycorrhiza have been reported to reduce the severity of disease caused by plant parasitic nematodes.
Most of arbuscular mycorrhiza (AM-fungi) and nematode interaction studies have been made with sedentary endoparasites, especially with the root-knot nematodes. Only little attention is made to the migratory endoparasitic nematodes, but it has already been shown that mycorrhizal plants decrease nematode reproduction compared with non-mycorrhizal plants. Different interaction mechanisms between mycorrhizal fungi and plant-parasitic nematodes are already suggested and will be discussed in paragraph 2.4.

2.3 Mycorrhizal fungi

2.3.1 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 in 1885 by the German plant pathologist A. B. Frank, literally means "fungus root". It therefore basically designates the symbiotic association between a fungus and the roots of a plant. 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. Contrary to endomycorrhizal fungi, the hyphae of ectomycorrhizal fungi cause only a small increment of the volume of soil exploited by the roots. Endomycorrhizal hyphae, however, grow both inter- and intracellularly in the root cortex, after penetration through the epidermis or root hairs. No fungal mantle is 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 endomycorrhizal association is the vesicular-arbuscular type, which produces fungal structures (arbuscules and sometimes vesicles) in the cortex region of the root. Arbuscular mycorrhizal fungi (AM-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 have been found all over the world in arctic, temperate and tropical regions, and from aquatic to desert environments. AM-fungi live in association with approximately 85 % of herbaceous plants, making mycorrhizal symbiosis a rule rather than an exception (Gerdemann, 1968).

2.3.2 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. AM-fungi simultaneously colonize the roots and the rhizosphere of plants and spread out over a few centimeters in the form of ramified filaments. This filamentous network, dispersed inside as well as outside the roots, allows the plant to acquire a greater quantity of water and soil

minerals, such as phosphorus, nitrogen, copper and zinc. The colonized plant is thus better nourished and can better adapt 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). In return, the fungus receives from the plant metabolized nutrients that it is unable to synthesize itself, such as carbohydrates, amino acids and secondary metabolites (Harley & Smith, 1983).

2.3.3 Taxonomy and morphology

The AM-fungi are recently grouped in a new phylum, the *Glomeromycota* (Schüβler *et al.*, 2001) and they all belong to the order of the *Glomerales*.
AM-fungi infection develops in two stages, an extraradical phase with extraradical hyphae and spores scattered in the surrounding soil, followed by an intraradical phase with intracellular unbranched hyphae, intracellular branched hyphae, intercellular hyphae, arbuscules, sometimes vesicles (Figure 2.8 A) and spores (Figure 2.8 B).

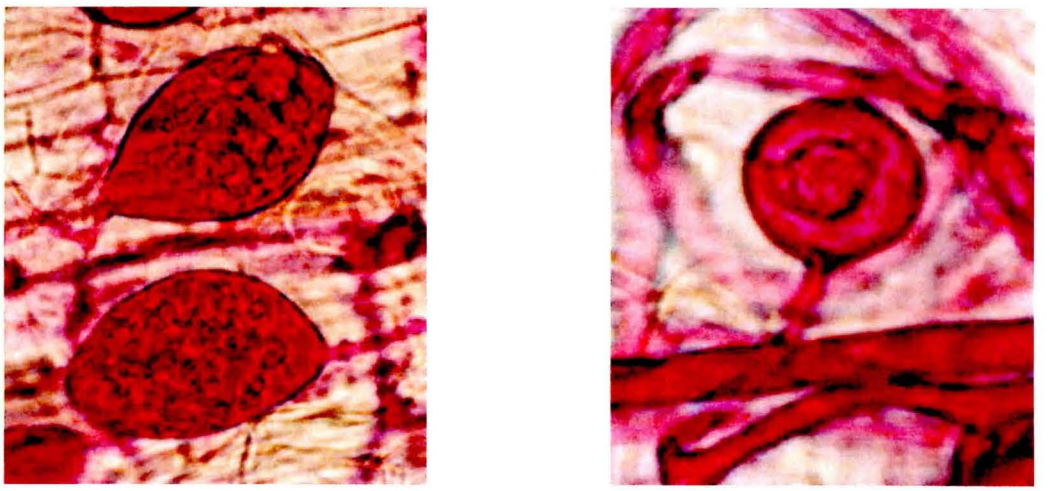


Figure 2.8: Detail of mycorrhizal structures colored with fuchsine acid under a 100 x magnification. Left: vesicles Right: spore

2.3.3.1 Extraradical phase

Spores are resting structures, with a diameter of 20 to 400 µm, that contain multiple nuclei and carbon storage compounds (glycogen and lipids) (Bonfante *et al.*, 1994). AM-fungal spores can germinate in absence of a host plant, but they are not capable of extensive independent mycelial growth and consequently of completion of their life cycle (Mosse, 1988). After germination of the spores, the hyphae grow in the soil, but only in the presence of signals released by host roots, morphogenetic differentiation of infection structures occurs, allowing a functional symbiosis to be established (Giovanetti *et al.*, 1994).

The skeleton of the extraradical mycelium consists of thick-walled 'runner hyphae' (Bago *et al.*, 1998). First order runner hyphae develop in a straight manner, branching shortly behind the apex, at an angle of approximately 45° and at regular intervals between 25 and 300 µm, to give rise to higher orders of progressively thinner runner hyphae, which extend the fungal colony radially. Second and higher orders of runner hyphae, also called 'absorptive hyphae' form small bunches of dichotomously branched hyphae at more or less regular intervals (Bago *et al.*, 1998). These structures are called 'branched absorbing structures, (BAS)'. They can appear alone, or associated with spores. These branched absorbing structures are sites of intense metabolic activities and seem to be the preferential sites for mineral nutrient acquisition by the extraradical mycelium (Bago *et al.*, 1998; Bago, 2000).

2.3.3.2 Intraradical phase

AM-fungi develop a major network of microscopic filaments in the soil. When filaments of the fungus organism come in contact with a young root, the extraradical hypha swells apically and increases in size forming an appressorium-like structure between epidermal cells. Root penetration occurs when hyphae from the appressoria penetrate epidermal or cortical cells. The outer cortical root layers are often colonized by intracellular hyphae, characterized by a linear or more often looped arrangement, without any sign of branching. When the fungus reaches the middle area of the cortical parenchyma, the fungus becomes intercellular. It grows longitudinally in the intercellular spaces of the middle root cortical cells and then extends deeper into inner cortical cell layers in a radial manner (Smith & Read, 1997). Intercellular hyphae are thick-walled and long-lived, and they seem to provide the living skeleton of a mycorrhizal infection unit. From these, lateral branches arise to form highly branched, terminal structures: the arbuscules.

Arbuscules start to form approximately 2 days after root penetration. They grow inside individual cells of the root cortex, but remain outside their cytoplasm, due to invagination of the plasma membrane. They increase the surface area of contact between the symbionts, and at arbuscular interfaces plant and fungal plasma membranes are in intimate proximity.

The arbuscule life span is limited to a few days, after which the arbuscule detoriorates and collapses. The structure of the arbuscule has lead to the general belief that arbuscules are the sites of carbon uptake by AM-fungi and of mineral nutrient export to the plant (Harley & Smith, 1983). This assumption is based upon ultrastructural studies, which revealed that arbuscules have a distribution of organelles typical of metabolically active cells (Bonfante-Fasolo, 1984). Some experiments conducted in the last decade however showed that the intercellular hyphae play a greater role in carbohydrate absorption, whereas the arbuscular branches would be mainly associated with the mineral nutrient transfer to the periarbuscular apoplast (Gianinazzi *et al.,* 1991). Published data showing convincingly the involvement of arbuscules in C acquisition in AM-fungi are still lacking (Bago, 2000).

At time of arbuscular formation or often shortly thereafter, some AM-fungi form inter- and/or intracellular vesicles. These globose bodies are caused by intercalary or terminal swelling of the hyphae and are found in inner and outer cortical parenchyma layers. Vesicles are thought to function as either storage organs of large quantities of lipids, or simply as resting organs. They continue to develop when the arbuscules senesce. During stress situations when a low supply of metabolites is received from the host plant, these reserves are used by the fungus and the vesicles consequently degenerate (Smith & Read, 1997).

Spores are also differentiated in the roots. They act as reserve and propagation organs. Figure 2.9 gives an overview of the different mycorrhizal structures inside and outside the root.

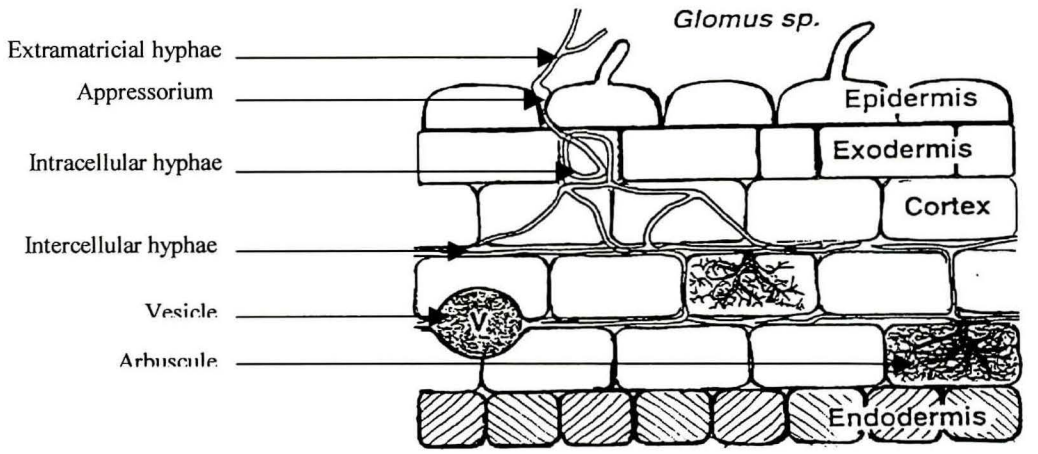


Figure 2.9: Main structures of a typical vesicular-arbuscular mycorrhizal infection (*Glomus* sp.) in a root (Powell & Bagyaraj, 1984).

Extension of the fungus in the roots and rhizosphere follows a sigmoidal curve on which three phases can be distinguished (Menge, 1984): an initial lag phase when primary infection takes place; an exponential phase when the fungus spreads rapidly in the root; and a plateau phase during which root and fungal growth proceed at the same rate. The time periods required for each of these phases differs among crops and mycorrhizal species.

2.3.4 Importance in agriculture

AM-fungi are renowned for their growth promoting properties, thought to be due to the increased nutrient levels found in mycorrhizal plants compared to non-mycorrhizal plants. Lin & Chang (1987) showed a positive effect of the AM-fungi on the P and N contents in the plant and an improvement of the vegetative development. This was also the result of studies performed by Lopez *et al.* (1997). The large hyphal network around the roots enlarges the absorbing surface so mycorrhizal plants can take up more nutrients from the soil than non-mycorrhizal plants (Sanders & Tinker, 1971). The extraradical hyphae and supposedly the BAS for a great extend, increase the plant access to soluble phosphates and other nutrients in the soil (Bago *et al.*, 1998; Jaizme-Vega, 1998). The external mycelium is able to take up phosphate away from the depletion zone around each root, translocate it, and release it in the cortex of the host root. This is due to the increased root volume and surface area, thus decreasing diffusion path length for

phosphate, and also to the capability of the small hyphae to penetrate into sites too small to be reached by plant roots. It is however unlikely that mycorrhizal roots have access to sources of soil phosphate other than the inorganic labile pool (Sanders & Tinker, 1971).

The AM-fungi affect not only plant development and nutrition, but can also increase the plant natural resistance to biotic (pathogens) or abiotic (hydric stress, salinity, pollution (i.e. heavy metals)) stresses (Hussey & Roncadori, 1982; Sylvia, 1989; Charest *et al.*, 1993).

The extraradical mycelium of AM-fungi also plays a structural role. It is an important binding agent and plays a fundamental role in soil conservation. The extraradical mycelium can improve the structure of the soil through the formation of stable aggregates by physical entanglement and the production of binding agents which increase its resistance to erosion (Varma, 1998; Dodd *et al.*, 2000).

Also in micropropagation practices, AM-fungi are of important interest. The pre-inoculation of micropropagated plantlets free of pathogens appears to be an appropriate management practice to introduce AM-fungi in the field (Delvaux *et al.*, 1998). Potential advantages are numerous. First, AM-fungi are already established in the roots and will consequently have a competitive advantage over soilborne pathogens. Second, higher plant growth rates may result in a shorter nursery phase. Third, far less AM propagules are required to inoculate young plantlets (having fewer roots) than older plants and may result in reduced costs (Jaizme-Vega *et al.*, 1997).

Although all the beneficial effects of AM-fungi on the growth and health of *in vitro* propagated banana plants have been recognized, their obligate biotrophic status, i.e. the fact that these fungi can not be cultivated in absence of a host plant, limits there large-scale production and reduces their potential use as an inoculum in agricultural practices. The inability to grow these obligate biotrophic fungi in axenic culture led to the development of monoxenic culture systems using transformed roots as a host partner (Declerck *et al.*, 1998). With *Agrobacterium rhizogenes* Riker (Ri T-DNA) transformed carrot roots as a host, large amounts of contaminant-free AM inoculum could be produced (Plenchette *et al.*, 1996). Recently, experiments with the monoxenically produced AM inoculum showed a significantly increased growth of micropropagated bananas (Declerck *et al.*, 2002).

The mutualistic symbiosis between the AM-fungi and a host plant is non-host specific (Gerdemann, 1968). However, experiments have shown that the efficiency of the symbiosis can differ between different fungus/host pairs (Declerck *et al.*, 1995). Selection

of efficient AM-fungus/banana cultivar couples for a given set of field conditions should thus be a consideration in the improvement of banana cropping systems.

2.4 Interactions between mycorrhiza and nematodes

Since plant-parasitic nematodes and AM-fungi are both present in the same structures of the root system of many plants, it was logical to consider a possible interaction between these two groups of organisms. The obligate symbiotic AM-fungi may stimulate plant growth whereas the obligate plant-parasitic nematodes usually suppress plant growth. The action of AM-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 AM-fungi and nematodes on various host plants 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 AM-fungi promote resistance or tolerance to nematodes in 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 crops this effect is significant enough to consider mycorrhizal infection as a more or less effective mean of biological control. In some studies however, mycorrhizae seem to have no effect (O'Bannon & Nemec, 1979; Thompson & Hussey, 1981; Pinochet *et al.*, 1995; Pinochet *et al.*, 1998) or sometimes even more nematodes were observed in the mycorrhizal roots (Kotcon *et al.*, 1985; 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. The difference in root colonization by the fungus also has a direct effect on the nematodes as they are thought to compete in space and resources. 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 were made to explain the beneficial effects of AM-fungi on plants parasitized by nematodes (Hussey & Roncadori, 1982; Smith, 1987) but the exact mechanisms that are responsible for resistance or tolerance to nematodes are not yet known. Possible hypotheses are:

Improvement of the nutrient status of the host plant. Since increased uptake of phosphorus and other nutrients and water results in more vigorous plants, the plant itself may thus be more tolerant to pathogen attack (Harley & Smith, 1983).

Physiological and biochemical changes. Cells colonized by the 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 mycorrhizal plants, affecting nematode feeding (Pinochet *et al.*, 1996). Also, the amino acid content of mycorrhizal plants seemed to be 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).
The sugar content of a plant is also 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.

Changes in root morphology. Some morphological changes of the root system could result in reduction of nematode penetration. Schonbeck (1979) showed that mycorrhizal plants have a stronger vascular system, which means greater mechanical strength to lower the effects of pathogens. AM-fungi also induce an increased branching of the root system, resulting in a relatively larger proportion of higher order roots (Hooker *et al.*, 1994). In addition, AM-fungi increase lignification and suberization processes in the roots of the host, resulting in a reduced penetration of plant parasitic pathogens (Smith, 1987).

Competition for host photosynthates. Both the AM-fungi and the root pathogens depend on host photosynthates for their growth and it has been shown that they compete for the carbon compounds in the root (Harley & Smith, 1983; Smith, 1987; Linderman, 1994). When AM-fungi have primary access to photosynthates, the higher demand may inhibit pathogen growth. However, there is little or no evidence that competition for carbon compounds is a generalized mechanism for pathogen biocontrol by AM-fungi (Azcón-Aguilar & Barea, 1996).

Competition for infection and colonization sites. Because AM-fungi and plant-parasitic nematodes occupy similar root tissues, direct competition for space has been postulated as a mechanism of pathogen inhibition by AM-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 AM-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).

Microbial changes in the mycorrhizosphere. AM-fungi physiologically alter or reduce the root exudates of the host plant. This causes qualitative and/or quantitative alterations in microbial populations in the rhizosphere. Dependent on the AM-fungus involved, different microbial shifts occur in the mycorhizosphere, leading to stimulation of certain components of the microbiota, which in turn may be antagonistic to root pathogens. 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.
More investigation however is needed to elucidate how microbiota-mediated changes may be involved in biological control by AM associations (Azcón-Aguilar & Barea, 1996).

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 AM-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).

Migatory endoparasitic nematodes are reported to have diverse actions over AM-fungi. A few cases describe the absence of interaction in which the development and colonization of the symbiont are not affected by the pathogen (Hussey & Roncadori, 1978; Camprubí *et al.*, 1993; Duponnois & Cadet, 1994; Jaizme-Vega *et al.*, 1997). However, the nematode can be detrimental to mycorrhizal root colonization or spore production. For example cortical tissue of citrus root, disrupted by *R. similis*, effected mycelial growth and therefore suppressed the beneficial influence of this symbiont (O'Bannon & Nemec,

1979). Umesh *et al.* (1988) observed a reduced root colonization of *G. fasciculatum* when plants were inoculated with *R. similis*, while the spore production was not affected. Pinochet *et al.* (1995) however recorded a significantly lower spore production of *G. intraradices* in the presence of the nematode *P. vulnus*. Pinochet *et al.* (1998) even made the observation that mycorrhizal colonization with *G. mosseae* or *G. intraradices* did not affect the number of nematodes per gram of root in plants infected with *P. vulnus* while in contrast, *P. vulnus* significantly decreased the percentage of mycorrhizal colonization. Whether the nematodes can suppress the mycorrhizal colonization or not can also depend on soil conditions. Carling *et al.* (1989) recorded no effect of *M. incognita* on the root colonization by AM-fungi at high P rates, but at the lowest P rates, the nematode stimulated sporulation of *G. margarita* and suppressed sporulation of *G. etunicatum*.

The main causes affecting the arbuscular phase and the production of storage structures (vesicles and internal spores) appears to be the reduction of space for fungal colonization (Smith, 1988) and a decrease in supply of carbohydrates in the roots (Wallace, 1987). Both are a result of the nematode feeding and migration within the cortical parenchyma, causing necrotic lesions.

3 Objectives of the study

Dependent on the cultivar, mycorrhizal strain and environmental (or experimental) conditions, the effectiveness of the AM symbiosis in its protection of the roots of banana against plant-parasitic nematodes can vary greatly. Furthermore, the effectiveness of this colonization can vary between nematode species and within a species.

Our objective was to obtain a better understanding in the interactions between AM-fungi and nematodes on banana as a host plant. We consequently designed experiments to separate the three steps that lead ultimately to the evaluation of this interaction. We investigated separately the attraction, penetration and reproduction of nematodes in banana roots where AM-fungal colonization was present or absent.

Firstly, we conducted an experiment to study the reproduction of different *R. similis* populations in the roots with or without AM-fungal colonization. Two populations with a difference in pathogenicity were used to investigate whether the influence of the AM-fungi on the nematodes differs with the pathogenicity of the population. The experiment was performed in pot-culture. An additional experiment was conducted to investigate whether the inoculation procedure of the AM-fungi has an effect on the colonization of the roots and on the plant growth characteristics. Mycorrhization of banana plantlets is performed at the beginning of the acclimatization phase so to obtain a procedure for rapid colonization of the roots by AM-fungi, before the plantlets are planted in the field, can be of economical benefit and beneficial in the protection against nematodes.

It is not yet clearly understood whether AM-fungi play a role in the attraction, penetration or reproduction of the nematodes in the roots. Therefore, a third experiment in pot-culture was conducted to study only the attraction and penetration of the nematodes to/into the roots of banana plantlets where AM-fungal colonization was either present or absent.

Thereafter, another experimental setup was developed to study the attraction and penetration of the nematodes to/into a reduced root system. Experiments were conducted in Petri dishes to obtain more controlled conditions. The setup consisted of an excised root, embedded in sand. Now, the exact localization of inoculation of the nematodes along the root could be chosen and the exact location of the penetration of the nematodes could be observed. The goal was to determine whether nematodes enter the root on the place where they are inoculated or whether they move along the root, up to a favorable or preferential part of the root to penetrate.

Finally, to decipher whether nematodes are attracted to the roots of banana, we designed an *in vitro* experiment that allowed to study the exact path of the nematodes from their inoculation point to the place where they enter the roots. For this experiment, only the

host plant (type of cultivar) and the nematode population could be assayed as up to now, *in vitro* mycorrhizal plants cannot be obtained. The goal of this experiment was to investigate whether there is a difference in attraction to the roots between populations with a different pathogenicity and whether two different cultivars have a different influence on the interaction of the nematodes to the roots.

4 Materials and methods

4.1 Overview of the experiments

A first set of experiments was performed in pot culture. In experiment 1, the penetration and reproduction of the burrowing nematode *R. similis* in the roots of banana plantlets, colonized with the AM-fungus *Glomus* sp. was investigated. The duration of this experiment was 11 weeks. In experiment 2 we tried to compare two different inoculation procedures of AM-fungi. In one case, the mycorrhizal inoculum was added in a layer, surrounded by two layers of substrate, while in the other case the mycorrhizal inoculum was homogenized within the substrate. The third experiment (experiment 3a and 3b) resembled experiment 1, only the time scale was different. The time between inoculation and observation was only 24 hours and/or 48 hours.

In a second set of experiments, we tried to develop more controlled conditions to investigate the initial attraction and/or penetration of *R. similis* to/into the roots of non-mycorrhized banana plantlets of different cultivars. These experiments were performed in Petri dishes.
In experiment 4, different parameters that could play a role in the initial attraction of nematodes to the roots of banana plantlets were examined. The different parameters we took into account were the inoculation procedure, the time interval between inoculation and observation, the segment of the root used and the humidity of the sand. In a last experiment (experiment 5), we investigated the effect of root exudates of different banana cultivars on different populations of *R. similis*.

4.2 Preparation of the experiments

4.2.1 Biological material

4.2.1.1 Banana cultivars

The banana cultivars used were Poyo (*Musa AAA* group, Cavendish subgroup), Yangambi km 5 (*Musa AAA* group, Ibota subgroup) and Obino l'Ewai (*Musa AAB* group, Plantain subgroup).
Poyo was chosen because of its susceptibility to banana nematodes, especially *R. similis* (Wehunt *et al.*, 1978; Price, 1994; Pinochet *et al.*, 1998). We used this cultivar in pot experiments and Petri dish experiments to investigate the interaction between different nematode populations and different strains of mycorrhiza, using the roots of this cultivar as a host. Obino l'Ewai is also very susceptible to nematodes (Blomme *et al.*, 2000) while Yangambi km 5 shows resistance or partial resistance to *R. similis* populations (Fogain & Gowen, 1998; Stoffelen, 2000). These two cultivars were used in Petri dish experiments to investigate the initial attraction and penetration of different nematode populations to/into the roots of these banana cultivars. Obino l'Ewai was also used in a pot experiment to assess the colonization of mycorrhiza in the roots of this cultivar.

4.2.1.2 Mycorrhizal strains

Three different mycorrhiza strains were used in the experiments. They respectively belong to the species *Glomus proliferum* (obtained from UCL, Belgium), *Glomus* spec. (obtained from Biorize®, France, code of the strain: DA) and *Glomus mosseae* (obtained from ICIA, Tenerife). In an additional test, a fourth strain was tested for its efficiency. This strain belongs to the species *Glomus intraradices* Schenck and Smith (obtained from IRTA, Spain).

4.2.1.3 Nematode species

The nematodes that were used in all the experiments belong to the species *R. similis.* The nematodes, used in the experiments where substrate or sand was used (no medium), were routinely cultured monoxenically on carrot discs (O'Bannon & Taylor, 1968) at CIRAD, Montpellier (France). Three different populations were used, originating from Cameroon, Ivory Coast and Australia. The original hosts of these populations were 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 (Sarah *et al.*, 1993; Fallas *et al.*, 1995; Hahn *et al.*, 1996) (see paragraph 2.2.2.3).

The nematodes used in the experiments where medium was used were cultured on alfalfa callus (Riedel, 1985 in Zuckerman *et al.*, 1990). Nematodes reared on carrot discs can cause bacterial contamination on nutrient media. Indeed, the carrot tissue is a source of contamination since the carrots are only surface-sterilized. Sterilization of the nematodes with streptomycin sulfate eliminates only the bacteria on the surface of the nematodes, but not in the digestive system. Therefore, nematodes from carrot discs need to be sterilized with $HgCl_2$ before they can be used on nutrient media. Since the sterilization method is toxic for humans and not always successful, alfalfa callus was used for the aseptic culturing of nematodes. This method is more sterile so the sterilization of the nematodes with $HgCl_2$ is not necessary. Culturing of nematodes on alfalfa callus is routinely performed at the Laboratory of Tropical Crop Improvement, K.U.Leuven (Belgium).
Two different populations were used. One originated from Uganda, the other from Indonesia. These two populations were chosen because the population of Uganda is highly pathogenic (Sarah *et al.*, 1993; Fallas *et al.*, 1995; Hahn *et al.*, 1996) (see paragraph 2.2.2.3) while the population of Indonesia has a low pathogenicity (Elbadri *et al.*, 2001).

4.2.2 Growth of plant material

4.2.2.1 Experiments performed at CIRAD, Montpellier, France

The banana plantlets of the cultivar Poyo were obtained through *in vitro* micropropagation by Vitropic®, Montpellier (France). The plantlets were delivered in boxes of 100 units, containing 100 ml of a growth medium (MS rooting, Murashige & Skoog, 1962) (see annex 2). At time of delivery, the plant height was around 6 cm and weighted between 0.5 and 2 g.

Preparation prior to planting involved washing the roots to remove the medium, removal of all the leaves except the two or three youngest and cutting the roots, except two or three, 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 AM-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 (7/8 Biot Sand, 1/8 soil) containing mycorrhizal roots. After 8 weeks, the roots of the leek were washed and chopped in 0.5 to 1 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 1 week in a tray with a plastic cover thus creating a 'micro greenhouse' until the end of the experiment and no 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 75 % and a 12 hour/12 hour photoperiod.

4.2.2.2 Pot-experiment performed at the Laboratory of Tropical Crop Improvement, K.U.Leuven, Belgium

The plantlets from the cultivars Yangambi km 5 and Obino l'Ewai were micropropagated *in vitro* in the Laboratory of Tropical Crop Improvement, K.U.Leuven (Belgium). *Musa* tissue culture plants from these cultivars were obtained from The International Musa germplasm collection at the INIBAP Transit Centre at K.U.Leuven (Belgium). Under the laminar flow, leaves, buds and roots of the plants were removed and shoot tips of about 1 cm long were excised. Each shoot tip was then transferred to a test tube containing 25 ml of MS rooting medium. The tubes were incubated at 28 °C with 16 hour photoperiod for 4 weeks.
After this period, the banana plantlets were taken out of the tubes and the medium was removed from the roots by rinsing them under running water. The plantlets were placed in a tray (29 cm by 42 cm, 8 cm deep) containing a substrate sterilized at 121 °C for 20 minutes. The substrate consisted of 1/3 sand and 2/3 soil containing 35 % organic matter and having a pH(H_2O) of 3.5-4. Each tray was divided in two parts by a partition. To one

part of the tray, mycorrhiza were added homogeneously; to a second part, mycorrhiza were added in a layer; another part served as control where no mycorrhiza were added. Each part contained several plantlets. The mycorrhizal inoculum was cultivated on sorghum and consisted of a mix of soil, roots and spores. The trays were then placed in a culture chamber with continuous light for 8 weeks. The first 3 weeks, they were covered with a plastic cap to maintain maximum humidity. Plants were watered when needed and no fertilizer was added.

4.2.2.3 Petri dish experiments performed at the Laboratory of Tropical Crop Improvement, K.U.Leuven, Belgium

The plantlets used for the Petri dish experiment were cultivated in the same way as described in paragraph 3.1.2.2. After the first 4 weeks in a test tube, the plantlets were transferred to Petri dishes containing MSR medium (see below).

4.2.3 Nematode cultures

A. Nematodes cultured on carrot discs

4.2.3.1 Culturing of nematodes

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

- 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 containing Streptomycine at 500 ppm. The flasks were placed in a box at 27 °C for 1 or 2 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. Therefore, the nematodes were recuperated from the jars with a Pasteur pipette with sterile water and brought into a conic tube. They were centrifuged for 3 minutes at 2500 rpm. The supernatant was removed and replaced by a solution of $HgCl_2$ (0.01 %). After, the tube again was homogenized and centrifuged for 3 minutes at 2500 rpm. Then, the supernatant was removed and replaced by sterile water to rinse the nematodes. This also was homogenized and centrifuged for 3 minutes at 2500 rpm. After, the supernatant was replaced by dihydrostreptomycine (0.2 %), homogenized and centrifuged, again for 3 minutes at 2500 rpm. The supernatant was for the last time replaced by water, homogenized and centrifuged for 3 minutes at 2500 rpm. The volume of water was then reduced to obtain a concentrated suspension of nematodes.

Three or four drops of this solution were added on top of the carrot discs (approximately 1000 nematodes per jar). 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 flow. 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.

4.2.3.2 Nematode collection from the carrot discs

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 to 3 ml of sterile water and a turn of the jar, the nematodes were suspended in the water. The nematodes were then pipetted and transferred to a graduated tube. Nematode concentration was assessed by counting, and dilution carried out if necessary.

4.2.3.3 Nematode counting procedure

The nematode suspension was shaken well to ensure a homogenized suspension. A sample was then pipetted into a counting cell and the number of nematodes per milliliter was assessed using a light microscope at 40 x 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 concentrations needed for the inoculations.

4.2.3.4 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.

4.2.3.5 Inoculation of banana roots in Petri dish, containing sand

Small Petri dishes (5.5 cm in diameter) were used for a new experimental setup. More controlled conditions were 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 g of sand (Fontainebleau Sand, granulometry of 230 to 310 μm) were added to the Petri dish.
The root fragments tested had a length of 4.5 cm and possessed either the meristem part of the root or not. Each root was placed in a Petri dish and an additional 15 g of sand was added. Six ml of sterile water were added to saturate the sand. The nematodes (200 or 400 per Petri dish) were suspended in 0.5 ml of water. This inoculum was added in different ways, depending on the experimental procedure (see paragraph 4.3.4). After inoculation, the Petri dishes were covered with their lids and the dishes were placed in a dark box at 27 °C for 24, 48 or 72 hours. The dishes were sealed with parafilm to retain humidity.

B. Nematodes cultured on alfalfa callus

4.2.3.6 Preparation of the callus and culturing of the nematodes

To obtain alfalfa callus from seeds, three steps had to be followed (Riedel, 1985 in Zuckerman *et al.*, 1990):

- the surface sterilization of the seeds;
- the germination of the seeds in a simple medium;
- the production of the callus on the modified White's medium.

The seeds were introduced for 15 minutes in 5 to 10 ml of a concentrated solution of H_2SO_4. They were rinsed with destilled water, and then soaked for 15 minutes in a $HgCl_2$ solution (1 ppm $HgCl_2$ in 30 % ethanol). After that, the seeds were rinsed with destilled water. For the germination of the seeds, Petri dishes (9 cm diameter) were filled with 25 ml of the germination medium:

- Sucrose	10 g	} in 1 l distilled water
- Difco Yeast extract	2 g	
- Agar	10 g	

The medium was autoclaved before use (121 °C for 20 minutes). The seeds were then spread on the Petri dishes (16 per dish). The dishes were sealed with parafilm and placed for 4 to 7 days in the dark at 22 to 24 °C.

For the production of the callus, modified White's medium, containing 2,4-D and α-NAA (Riedel & Foster, 1970) was needed (see annex 3). After the medium was autoclaved and the tubes were filled (14 ml/tube), the tubes were placed on an incline of 45 ° to cool down in order to make slants. Sixteen seedlings were transferred per slant. The tubes were then put in the dark at 21 °C for 7 to 10 days to allow the callus to develop. After the callus had developed, it was transferred to Petri dishes with White's medium for rearing the migratory nematodes. For inoculation, selected females were sucked with a micropipette and placed directly on the surface of the callus. Inoculum density was around 25 females per plate.

4.2.3.7 Nematode collection

For the extraction of the nematodes from the medium or callus, the modified Baerman funnel technique was used (Hooper, 1990). Small slices of medium of about 1 cm^2 or little pieces of callus were aseptically transferred into a sterile 70 µm pore sieve. The sieve was placed on a watch glass with a few drops of sterile distilled water in a parafilm sealed Petri dish. These were stored in the dark at 27 °C for one night. Overnight,

nematodes migrate from the callus or medium into the water. The suspension only consists of living nematodes making it useful for inoculation.

4.2.3.8 Inoculation of banana roots in Petri dish containing MSR (Modified Strullu Romand) medium

Petri dishes with a diameter of 13.5 cm were used for this experiment. With this setup, the initial attraction of nematodes to the roots right after inoculation could be assessed in more controlled conditions. The whole experimental setup had to be performed under laminar flow. Petri dishes were filled with 100 ml MSR medium (Modified Strullu Romand medium, see annex 4) (Diop, 1995). The banana plantlets were taken out of the tubes after 4 weeks of rooting and the medium was removed from the roots as much as possible. Plants were then laid on the medium in the Petri dish, spreading the roots as far as possible. The roots were slightly pressed into the medium without damaging the medium too much (Figure 4.1). The Petri dishes were sealed with parafilm and placed in a dark box for 24 hours at 26 °C to allow diffusion of root exudates in the medium.

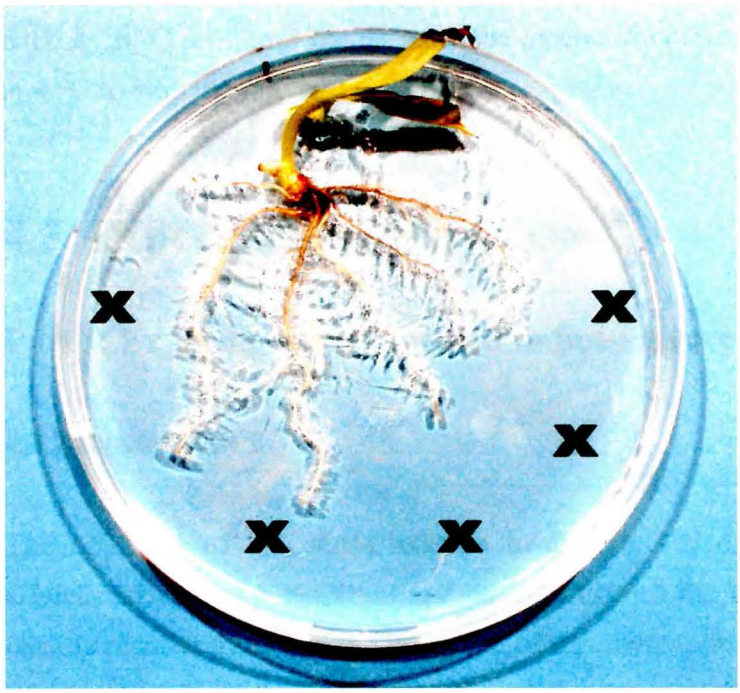


Figure 4.1: Four weeks after the micropropagation, the banana plantlets were placed in Petri dishes (diameter 13.5 cm) filled with 100 ml MSR (Modified Strullu Romand) medium. Five nematodes were inoculated on the medium, as far as possible from the roots (X).

Prior to inoculation with nematodes, a micropipette had to be made. For this, tips of two pre-sterilized Pasteur pipettes were pressed against each other in a flame. When their tips had melted, they were pulled away quickly from each other to create small diameter openings. The too small and too sharp ends of the pipettes were cut with a sterile scalpel. A rubber top was placed on the pipette and the middle portion of the pipette was heated to create a vacuum that would facilitate aspiration. To select the nematodes for inoculation, the watch glass, containing the nematodes, was placed under the light microscope (under the laminar flow) and observed at 40 x magnification.

In each Petri dish, five drops of the suspension, with one nematode per drop, were placed on the surface of the medium, as far as possible from the roots, but not too close to the Petri dish wall (Figure 4.1). Inoculation of a nematode too close to the wall opens the possibility that the nematode will crawl to the side, out of the medium. It was also tried to inoculate the nematodes on the same distance from the roots, giving them all the same chance to reach a root. It is important after inoculation to let the Petri dish open for about 5 minutes so the drop of water can evaporate. If the nematode stays embedded in the drop of water, it can not make a track in the medium so detecting the movement of the nematode will be impossible. After inoculation, the Petri dishes were incubated in the dark at 26 °C for 2 or 3 hours. After the incubation period, the plates were placed in a box at 4 °C to stop the movement of the nematodes.

4.3 Experimental procedure

Part I: Experiments performed in pot-culture

4.3.1 Experiment 1

The objective of this study was to investigate the effect of the mycorrhizal fungus *Glomus* sp. on the penetration and reproduction of the burrowing nematode *R. similis* in banana plantlet roots and the influence of this nematode on the colonization effectiveness of the mycorrhizal strain. The duration of the experiment was 11 weeks.

At planting, 50 % of the plants were mycorrhized with *Glomus* sp.; the other 50 % remained non-mycorrhized. After 4 weeks, the plants were transferred to square pots (850 ml; 7.5 cm at the base, 10.5 cm at the top) containing 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 *R. similis* from Australia or a population from Ivory Coast. A treatment without nematodes was also used in these experiments.
The different treatments were:

AM-fungi	+ nematodes Australia	**No AM-fungi**	+ nematodes Australia
	+ nematodes Ivory Coast		+ nematodes Ivory Coast
	no nematodes		no nematodes

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 plants were placed in a completely random design. Nematode attraction, penetration and multiplication were investigated 5 weeks after inoculation. Mycorrhizal root colonization was assessed at the end of the experiment, 11 weeks after planting (see paragraph 4.4.4). Figure 4.2 gives an overview of the different steps conducted in this experiment.

Week 1	Planting of the micropropagated banana plantlets in pots of 250 ml containing substrate (1/8 soil, 7/8 Biot sand).
	Adding of mycorrhizal inoculum (*Glomus* sp.) to 50 % of the plantlets.
Week 5	Transfer of all the plants to pots of 850 ml.
Week 7	Inoculation with different nematode populations (Australia or Ivory Coast), 200 nematodes/pot or no inoculation.
Week 11	Observation and analysis.

Figure 4.2: Overview of the different steps conducted in the first pot experiment (experiment 1).

4.3.2 Experiment 2

In this experiment, two different inoculation procedures of mycorrhiza (*G. mosseae*) were compared. The idea comes from the fact that both procedures are used in recognized laboratories.

At planting, mycorrhiza were added to the substrate. In one part of the tray (eight replicates), the mycorrhiza were inoculated in layers: a layer of substrate was put in the tray to cover the bottom, then a layer of mycorrhiza (a mix of soil, roots and spores) was added and after that, another layer of substrate was added.
In the other part of the tray (also eight replicates), the mycorrhizal inoculum was homogenized within the substrate. A control was made without mycorrhiza (six replicates). Mycorrhizal root colonization was assessed 8 weeks after planting.

4.3.3 Experiment 3

In these experiments, we investigated the effect of mycorrhization (*G. proliferum* and *Glomus* sp.) on the attraction and penetration of the burrowing nematode *R. similis* to/into banana plantlet roots. The duration of these experiments was either 24 hours or 48 hours.

4.3.3.1 Experiment 3a (24 hours)

Fifty percent of the plants were inoculated with *G. proliferum*, the other 50 % remained non-mycorrhized. After 10 weeks, the plants were inoculated with two different nematode populations: a population from Australia and a population from Cameroon.
The different treatments were:

AM-fungi:	+ nematodes Australia	**No AM-fungi:**	+ nematodes Australia
	+ nematodes Cameroon 12		+ nematodes Cameroon 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 4.4.2). Mycorrhiza root colonization was assessed at the end of the experiment (see paragraph 4.4.4). Figure 4.3 gives a schematic overview of the different steps of this experiment.

Day 1	Planting of the micropropagated banana plantlets in pots of 250 ml containing substrate (1/8 soil, 7/8 Biot sand).
	Adding of mycorrhiza inoculum (*Glomus proliferum*) to 50 % of the plantlets.
Day 70	Inoculation with different nematode populations (Australia or Cameroon 12), 200 nematodes/pot.
Day 71	Observation and analysis.

Figure 4.3: Overview of the different steps conducted in the first short-time pot experiment (experiment 3a).

4.3.3.2 Experiment 3b (24 and 48 hours)

The mycorrhizal strain used in this experiment was *Glomus* sp. After 9 weeks, plants were inoculated with two different *R. similis* populations. A population originating from Australia, having a low pathogenicity, or a population originating from Cameroon, having a high pathogenicity. Four hundred nematodes suspended in 2 ml of sterile water were inoculated to the plants. Six replicates were made for each treatment. After 24 hours or 48 hours, the roots were stained (see staining procedure, paragraph 4.4.2) to investigate the nematode penetration. Mycorrhizal root colonization was also assessed. In figure 4.4, the different steps performed in this experiment are presented.

Day 1	Planting of the micropropagated banana plantlets in pots of 250 ml containing substrate (1/8 soil, 7/8 Biot sand). Adding of mycorrhizal inoculum (*Glomus* sp.) to 50 % of the plantlets.
Day 63	Inoculation with different nematode populations (Australia or Cameroon 12), 400 nematodes/pot.
Day 64	Observation and analysis of the first part of the experiment (24 h).
Day 65	Observation and analysis of the second part of the experiment (48 h).

Figure 4.4: Overview of the different steps conducted in the second short-time pot experiment (experiment 3b).

Part II: Experiments performend in Petri dish

4.3.4 Experiment 4

In this experiment, only non-mycorrhizal roots were used to study the initial penetration of the nematodes into the roots. Parameters 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 to use, and the humidity of the sand.

4.3.4.1 Experiment 4a

Two different inoculum densities and three time intervals between inoculation and observation were investigated.

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: two curves (3 mm deep) were made on both sides along the root at a distance of 2 mm from the root. The inoculum (two times 200 nematodes) was inserted in these curves.

For these experiments, root fragments including the meristem were used. The *R. similis* population used was Cameroon 12. Five replicates were made. Nematode penetration was investigated (after staining, see staining procedure, paragraph 4.4.2) at three time intervals, 24 hours, 48 hours and 72 hours after the inoculation.

4.3.4.2 Experiment 4b

In a first treatment the nematode penetration in root fragments including the meristem (meristem +) was compared with the nematode penetration in root fragments devoid of the meristem (meristem -). In another treatment, the impact of the humidity was tested. Respectively 5.5 ml and 6 ml of water were added to the sand in the Petri dish. Nematodes were inoculated at a quantity of two times 200 nematodes (see third inoculation procedure of experiment 4a).
Treatments:

- meristem - : roots devoid of apical meristem (the root was cut at least 4 cm from the meristem);
- meristem + : roots with apical meristem;
- humidity 5.5 ml: in stead of adding 6 ml of sterile water, only 5.5 ml was added (the sand was not saturated);
- humidity 6 ml: the usual 6 ml of sterile water was added (the sand was saturated).

For the humidity treatment the root fragment included the apical meristem. The population used was *R. similis* from Ivory Coast. Eight replicates were made. After 72 hours, the roots were stained for the observation of the nematode penetration (see staining procedure, paragraph 4.2.2).

4.3.4.3 Experiment 4c

The aim of this experiment was to investigate whether there is a difference in the release of root exudates after the roots have been cut. In the treatment 'storage 24 h', the Petri dish with the root was stored for 24 hours in a box at 27 °C before adding the inoculum.

The density of nematodes inoculated was two times 200 nematodes on both sides of the root (see experiment 4a).

Treatments:

- storage 24 h: 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;
- storage 0 h: The roots were inoculated directly after the roots were placed in the Petri dishes.

The root fragments used in these experiments possessed the apical meristem. A nematode population of *R. similis* from Ivory Coast was used. Eight replicates were made. After 48 hours, the roots were stained for the observation of the nematode penetration.

4.3.4.4 Experiment 4d

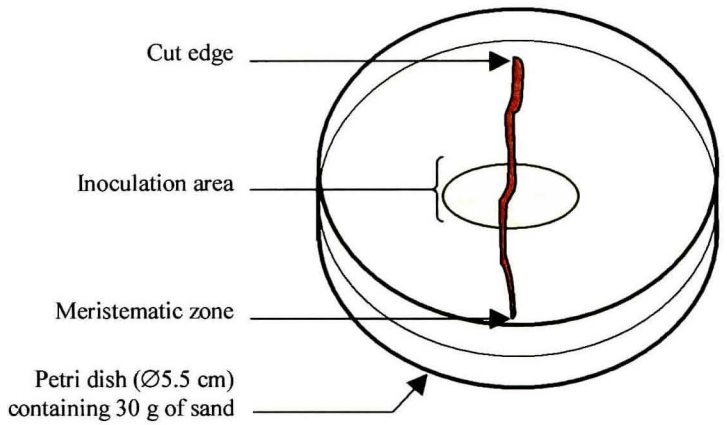
The place of adding the inoculum was investigated in this experiment (Figure 4.4). The density of the inoculum was 400 nematodes.

Treatments:

- inoculation half: The nematodes were inoculated in the middle of the root (Figure 4.5 A);
- inoculation meristem: The nematodes were inoculated on the meristem part of the root (Figure 4.5 B).

All the root fragments used in these experiments possessed the apical meristem. The population of *R. similis* was from Ivory Coast. Eight replicates were made. 72 hours after inoculation, the roots were stained for the observation of the nematode penetration (see staining procedure, paragraph 4.2.2).

A



B

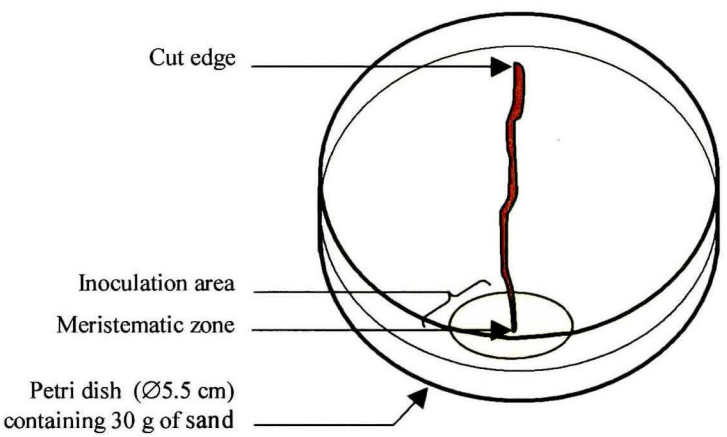


Figure 4.5: Petri dishes containing 30 g of sand and 6 ml of water. The meristem part of a root was brought in under the sand surface. Four hundred nematodes, suspended in 0.5 ml of water were inoculated on different places on the root. (A) inoculation in the middle of the root, (B) inoculation on the apical meristem.

4.3.5 Experiment 5

In this experiment, the initial attraction of the burrowing nematode *R. similis* to roots of banana and the effect of root exudates of different cultivars was investigated.
Only non-mycorrhized roots were used in this experimental setup.

Two different nematode populations (Uganda and Indonesia) and two different banana cultivars (Yangambi km 5 and Obino l'Ewai) were used. The attraction was investigated after two time-intervals.
The different treatments were:

Obino l'Ewai:	Uganda	2 h	**Yangambi:**	Uganda	2 h
	Uganda	3 h		Uganda	3 h
	Indonesia	2 h		Indonesia	2 h
	Indonesia	3 h		Indonesia	3 h

Five nematodes were inoculated in each Petri dish, in distant places on the plate, and as far as possible from the roots, but not too close to the wall. Five replicates were made for each treatment. Nematode attraction was investigated 2 or 3 hours after inoculation. Figure 4.6 gives an overview of the different steps of this experiment.

0 h	Transfer of the micropropagated plantlets to Petri dishes containing MSR medium.
24 h	Inoculation of the Petri dishes with different nematodes populations (Uganda of Indonesia).
26 h	Interruption of the movement of the nematodes of 50 % of the Petri dishes by placing them in the dark at 4 °C.
27 h	Interruption of the movement of the nematodes of the other 50 % of the Petri dishes by placing them in the dark at 4 °C.

Figure 4.6: Overview of the different steps conducted in experiment 5.

4.4 Procedures for observation and analysis

4.4.1 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 5. This method uses the difference in density between the nematodes and their surrounding vegetative matter to obtain their separation. Of five replicates of the mycorrhiza and nematode treatments, half of the roots were used to estimate the mycorrhizal colonization (see below). Of the treatment without nematodes, half of the roots of the 10 replicates were used for the mycorrhizal estimation. After the extraction, the number of nematodes for each sample was determined and the amount of nematodes per gram of root was calculated.

4.4.2 Nematode and mycorrhiza staining procedure

The nematodes and mycorrhiza staining procedure 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 organisms.

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 or until they had lost their dark pigmentation. Following this, the roots were thoroughly rinsed with water and placed into a hot (90 °C) staining solution for 1 hour. This staining solution consists of 50 % (v/v) lactic acid, 25 % (v/v) glycerol, 25 % (v/v) sterile water and 0.1 % (w/v) of fuchsine acid. The roots were then transferred to a destaining solution, which is identical to the staining solution, except that it does not contain fuchsine acid. After ± 1 hour in the destaining solution, the roots were ready for observation (Figure 4.7).

Figure 4.7: *Radopholus similis* in a root tissue of banana, colored by fuchsine acid and observed at a 80 x magnification.

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

For the observation of the roots from experiment 2 and experiment 4, 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; the other slide consisted of a thin plastic film.
A grid of 1.5 cm by 1.5 cm was made on the bottom slide to facilitate the observation of the roots. Thanks to this grid, the exact place of penetration of the nematodes could be determined so the exact penetration of the nematodes in different roots could be compared. Nematode penetration was investigated using a light microscope at 40 x magnification and the number of nematodes that penetrated in every segment of 1.5 cm of the root was counted. An estimation of the mycorrhizal colonization was made and the inter-relationship between the nematodes and the mycorrhiza examinated.

4.4.4 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 to 1 cm were randomly pressed on 9-cm-diameter round slides (see above) on which a 1 cm square grid is drawn

(Figure 4.8) and studied under the light microscope at 40 x magnification. At each point where the root was found to intersect a gridline, an assessment of mycorrhizal colonization was made (presence of mycorrhiza or not). In these experiments, the intensity of the presence of the different mycorrhizal structures (hyphae, vesicles or arbuscules) was not conducted. At least 100 intersections were studied and recorded for each root sample. The frequency 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.

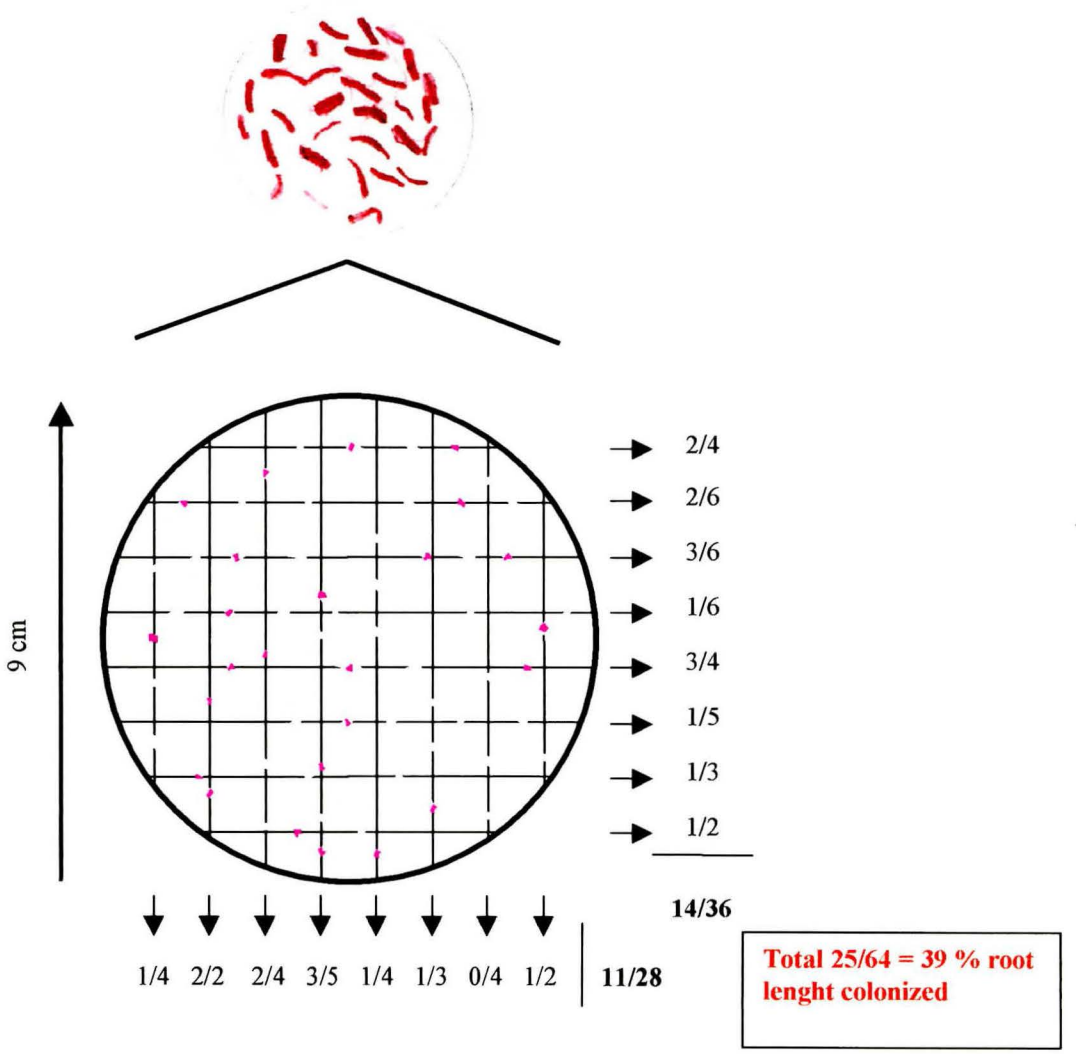


Figure 4.8: Schematic representation of a gridline intersect slide used to estimate mycorrhizal colonization. Roots are placed randomly on the side and colonization was checked at each intersection between the grid graduation and a root (described by Giovanetti & Mosse (1980) and reviewed by Brundrett *et al.* (1994)). The red clot on the pink roots represent presence of mycorrhiza on the intersections.

4.4.5 Visualization of the movement of the nematodes in the MSR medium

To investigate the attraction of nematodes with a difference in pathogenicity, to the roots of banana plantlets with a different nematode susceptibility, the movement of the nematodes to the roots was assessed. To visualize this, the Petri dishes were observed under the light microscope at 20 x magnification. When the nematodes started to move, they caused a little push into the medium. Because of this, their path became visible under a light microscope (or even with the naked eye).

An easier but more expensive method for the visualization of the nematodes was to make a print of the Petri dish on a contact film. By means of an enlarger with diaphragm-opening of 16, the Petri dish (without lid), put on a 'Kodalith Ortho Film type 3' was lighted for 3 seconds. The film was developed by putting it in a bath of 'Kodalith Developer and Replenisher' for 3 minutes; then in a solution of 6 % acetic acid for 10 seconds; for the fixation, the film was put in a bath of 'Kodak RA 3000 Fixer and Replenisher' for 2 minutes. Finally the film was rinsed under running water for 10 minutes and submerged in a water bath with 'Dreft' for 30 seconds.
On the photo, track appears transparent, while the medium appears black (Figure 4.9).

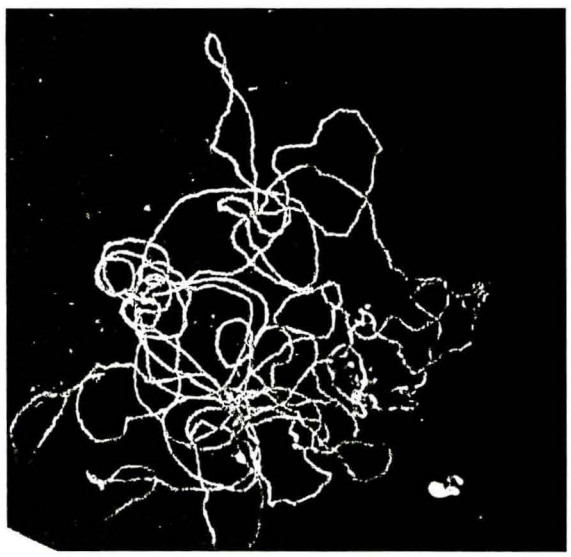


Figure 4.9: Path of a nematode (*Radopholus similis*) in MSR medium, 3 hours after inoculation. Visualized by a method described in paragraph 4.4.5.

4.4.6 Statistical analysis

4.4.6.1 Data

The experimental data were arranged in groups based on the independent variables. The groups are compared regarding the dependent variable with analysis of variance (ANOVA). All data analysis was performed with the statistical package STATISTICA® (Anonymous, 1997).

4.4.6.2 Assumptions of Analysis of variance

The interpretation of ANOVA is valid only when certain assumptions concerning the data are met: (i) the variances of the different groups are homogenous and (ii) the dependent variable is normally distributed within groups. Failure to meet these assumptions affects both the level of significance and the sensitivity of the F-test in ANOVA.
The homogeneity of the variances of the groups was tested with the Levene's test. Data with heterogeneous variances were transformed $\log_{10}(x+1)$ (mostly for nematode populations) or arcsin(x/100) (mostly for percentages) to obtain homogeneity of variances. If $P \geq 0.01$, homogeneity can be accepted. To check if the dependent variable is normally distributed within groups, the standardized residuals were calculated to determine occasional outliers. Standardized residuals were calculated for each observation as followed:

residual = (observation – means of group)

standardized residual = residual/standard deviation for all groups.

Outliers were defined as data with a standardized residual that fall outside the range from –2 to 2.

4.4.6.3 One way-ANOVA

Data that were normally distributed and had homogeneous variances were subjected to ANOVA. Single-factor ANOVA was used when the effect of one factor was investigated. The F-statistics revealed whether the means across the groups were statistically significant at $P \leq 0.05$ or not. If statistical difference was determined, multiple comparisons of means were used to identify the significant differences between the individual groups (means). Therefore, the Tukey HSD (Honest Significant Difference) test was used for equal and unequal group sizes.

4.4.6.4 Two way-ANOVA

When plants were simultaneously exposed to 2 factors, two-factor ANOVA was used. Before the effect of factor A and B could be determined, it was needed to determine if both factors interact or not. Two factors are said to interact if the effect of one factor changes as the level of the other factor changes. Interaction could be recognized by the F statistics of two-factor ANOVA. If the factors did not interact, the two main effects of factor A and B were determined separately. The F-statistics determined whether there was a significant effect of factor A and/or factor B. Tukey HSD test identified the effect of each factor in detail. If there was important interaction between both factors, the 2 effects were analyzed jointly.

5 Results and discussion

Part I: Experiments performed in pot-culture

5.1 Interaction between *Glomus* sp. and *Radopholus similis* on the *Musa* cultivar Poyo.

The aim of this experiment was to study the effect of the mycorrhizal fungus *Glomus* sp. on the penetration and reproduction of the burrowing nematode *R. similis* on the *Musa* cultivar Poyo. On the other hand, the influence of *R. similis* on the colonization effectiveness of the mycorrhizal strain was investigated.

5.1.1 Results

In this experiment, performed in pot-culture, young banana plantlets were inoculated with nematodes. The roots of these plantlets were either colonized by a mycorrhizal fungus (*Glomus* sp.) or left without mycorrhiza. Different *R. similis* populations were used, a population originating from Ivory Coast with a high pathogenicity and a population originating from Australia with a low pathogenicity.

Regarding the plant growth characteristics, no interactions nor main effects could be observed between the two independent variables (mycorrhizal and non-mycorrhizal plants/nematode population) in all the treatments (Table 5.1).
For the total number of nematodes recovered from the roots, also no interaction could be found, but two main effects could be observed (Table 5.2). On one hand, a significantly ($P \leq 0.05$) higher number of nematodes was recovered from the roots in the non-mycorrhized plants, compared to the mycorrhized plants. On the other hand, a significantly ($P \leq 0.05$) higher number of nematodes from the population originating from Ivory Coast was recovered from the roots, compared to the population originating from Australia. The same results were obtained for the total number of nematodes recovered from the roots and corm. Regarding the total number of nematodes extracted from the corm, a significantly ($P \leq 0.05$) higher number of nematodes of the population from Ivory Coast was extracted from the corm, compared to the number of nematodes of the population from Australia.
Looking at the number of nematodes per gram of root, also only a significant ($P \leq 0.001$) difference could be observed between the different nematode populations used in the

experiment. There was no significant difference between the mycorrhized and the non-mycorrhized plants.

For the reproduction rate, two main effects could be observed again: there was both a significant ($P \leq 0.05$) difference between the two nematode populations used, and between the mycorrhized and the non-mycorrhized plants. The reproduction rate was significantly higher in the non-mycorrhized plants compared to the mycorrhized plants, and for the population originating from Ivory Coast, compared to the population originating from Australia.

Table 5.1: Growth response of 'Poyo' banana to inoculation with *Glomus* sp. and two different populations of *Radopholus similis*, 11 weeks after inoculation with the AM-fungus (AMF) and 5 weeks after inoculation with 200 nematodes per plant.

Treatment		Plant height (cm)		Fresh shoot weight (g)		Fresh root weight (g)	
- AMF	Australia	5,90 ± 0,44	a	5,70 ± 0,91	a	4,80 ± 1,27	a
	Ivory Coast	6,30 ± 0,48	a	5,84 ± 1,10	a	4,77 ± 1,02	a
	Control	5,88 ± 0,53	a	5,59 ± 1,08	a	4,35 ± 1,41	a
+ AMF	Australia	5,81 ± 0,64	a	4,81 ± 0,84	a	4,87 ± 1,46	a
	Ivory Coast	5,86 ± 0,43	a	5,47 ± 0,98	a	4,42 ± 1,74	a
	Control	6,10 ± 0,69	a	5,98 ± 0,89	a	4,83 ± 1,08	a

Data are means of ten replicates. Means in the same columns followed by the same letter do not differ according to Tukey's test at $P \leq 0.05$.

Table 5.2: Reproduction of two different *Radopholus similis* populations in 'Poyo' banana with or without *Glomus* sp., 11 weeks after inoculation with the AM-fungus (AMF) and 5 weeks after inoculation with 200 nematodes per plant.

Treatment		Total nematodes in roots		Total nematodes in corm		Total nematodes (roots+corm) (=Pf)	
- AMF	Australia	862 ± 381	A	105 ± 97	A	968 ± 325	A
	Ivory Coast	2145 ± 1523	B	170 ± 161	B	2327 ± 1416	B
+ AMF	Australia	762 ± 625	A	54 ± 37	A	821 ± 623	A
	Ivory Coast	1339 ± 896	B	145 ± 125	B	1470 ± 888	B
		*		n.s.		*	

Treatment		Nematodes /g root		Reproduction rate (=Pf/Pi)		n
- AMF	Australia	1801 ± 49	A	4,8 ± 1,6	A	10
	Ivory Coast	434 ± 222	B	11,6 ± 7,1	B	10
+ AMF	Australia	172 ± 70	A	3,7 ± 3,2	A	10
	Ivory Coast	382 ± 186	B	7,4 ± 4,4	B	10
		n.s.		*		

Pi: Initial population inoculated to the roots. Data are means of ten replicates. All nematode densities were $\log_{10}(x+1)$ transformed prior to analysis. Capital letters indicate a significant ($P \leq 0.05$) main effect of the treatment nematode population. * indicates a significant ($P \leq 0.05$) main effect of the treatment mycorrhiza. Means are separated using the Tukey test ($P \leq 0.05$); n.s. indicates no significant difference according to the Tukey test ($P \leq 0.05$).

Regarding the percentage of roots colonized by the mycorrhizal fungus, 11 weeks after inoculation of the fungus, no significant difference could be observed between the different treatments. In roots inoculated with a *R. similis* population originating from Ivory Coast, 42.1 % of the roots was colonized with mycorrhiza. In the roots inoculated with a population originating from Australia, a colonization of 30.3 % was observed, while in the treatment without nematodes (Control), mycorrhizal colonization was present in 30.4 % of the roots (Table 5.3).

Table 5.3: 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	Amount of intersections mycorrhized[z]	% Colonization
Australia	188/632	30,3 ± 18,4 a
Ivory Coast	287/680	42,1 ± 14,7 a
Control	394/1321	30,4 ± 17,9 a

Data of the treatments 'Australia' and 'Ivory Coast' are means of five replicates, data of the treatment 'control' is a mean of ten replicates. All percentages were arcsin (x/100) transformed prior to analysis. Means in the same columns followed by the same letter do not differ according to Tukey's test at $P \leq 0.05$.
[z]The number after the slash indicates the amount of intersections on the slide; the number before the slash is the amount of intersections where a mycorrhizal structure could be observed.

5.1.2 Discussion

5.1.2.1 Plant growth characteristics

The very low plant height, dry shoot weight and dry root weight recorded is probably 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 fertilizers were added. This poor substrate was chosen to have a low level of phosphorus, as mycorrhizal root development is generally inhibited in soils with high P-contents (Gerdemann, 1968; Hussey & Roncadori, 1982; Saleh & Sikora, 1984; Smith *et al.*, 1986 a, 1986 b; Smith, 1988). In very phosphorus deficient soils the percentage of mycorrhizal infection is generally lower than in soils where some phosphorous is added or present, but at high phosphate levels, mycorrhizal infections die out (Mosse, 1981).
The lack of significant difference between plant height, dry root weight and dry shoot weight can be due to the fact that the first weeks after infection of the roots by the mycorrhiza, the mycorrhiza need a lot of carbohydrates to develop. Because of that, 10 % of the carbon (C) that is translocated to the roots pass to the fungal partner, making this C not available for the host plant (Fitter, 1991; Varma, 1998). Growth of the mycorrhized plants is thus limited. In order to improve plant growth, the benefits from a mycorrhizal association must be accompanied by a stimulation of photosynthetic C uptake, that will at least compensate the C lost to the fungus. It is generally assumed that enhancement of

photosynthetic rates results from increased levels of leaf P as a result of the mycorrhizal contribution to the P plant uptake (Fitter, 1991). However, such stimulation of photosynthesis will also depend on environmental factors such as atmospheric CO_2 and incident light levels (Louche-Tessandier *et al.*, 1999). For the host plant, these factors can therefore modify the balance between costs and benefits of a mycorrhizal relationship. After several weeks, the phenomenon of reduced growth of the mycorrhized plants dies out, making those plants growing faster than the non-mycorrhized plants. The time before differences in growth response between mycorrhized and non-mycorrhized roots can be observed depends thus on the host-fungal relationship and the environmental factors. In our experiment, no differences in growth response could be observed after 11 weeks, meaning that the mycorrhized plants probably just left the stage of growth reduction and started to build up a better growth response, thanks to the increased nutrient level brought about by the mycorrhiza.

Declerck *et al.* (1995) investigated the plant growth characteristics and the frequency of colonization of seven banana cultivars in combination with two *Glomus* species. In this experiment, they observed that a low growth response of the mycorrhizal plants compared to the non-mycorrhizal plants is possible, even if the root colonization is relatively high. This can be caused by a low relative mycorrhizal dependency (RMD) of the host plant.

Mycorrhizal dependency has been defined by Gerdemann (1975) as "the degree to which a plant is dependent on the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility". The magnitude of response is known to vary both between and within species (Plenchette *et al.*, 1983). The RMD of banana cultivars was determined by expressing the difference between the dry weight of the mycorrhizal plant and the dry weight of the nonmycorrhizal plant as a percentage of the dry weight of the mycorrhizal plant (Plenchette *et al.*, 1983). The value of dependency is 100 % when the plant fails to grow without mycorrhizae and 0 % when the difference between the dry mass of the mycorrhizal plant and the dry mass of the non-mycorrhizal plant is zero or not significantly different from zero (Plenchette *et al.*, 1983). Declerck *et al.* (1995) measured the RMD for seven banana cultivars in combination with *G. macrocarpum* and *G. mosseae* and obtained the lowest mycorrhizal dependency for the cultivar Poyo (12% with inoculation with *Glomus macrocarpum* and 0 % with *Glomus mosseae*). The *Glomus* species used by Declerck *et al.* (1995), however, are different than the *Glomus* species used in this experiment. It can thus only be assumed that the interaction between *Glomus* sp. and Poyo will give a low RMD. But we can also believe that, as equal

host/mycorrhiza couples can give a different colonization effectiveness when grown in different environmental conditions (Jaizme-Vega, pers. communic.), it is rather difficult to make any assumptions on this point.

Another possible explanation for the lack of significant differences in plant growth, dry root weight and dry root weight can be the too low root colonization of the fungus to make the symbiosis really effective (see below).

5.1.2.2 Efficiency of the root colonization

For the number of nematodes extracted from the roots, measured per gram of root, no significant difference could be observed between the mycorrhizal treatment and the non-mycorrhizal treatment, although this difference was significant regarding the total number of nematodes recovered from the roots. This is probably due to the high variability in the data obtained in this experiment (very high standard deviation). Regarding the total number of nematodes recovered from the roots, we could observe that the highly pathogenic population, originating from Ivory Coast, experienced more influence from the presence of AM-fungi compared to the low pathogenic population from Australia.
The reason that the presence of mycorrhiza could not induce a significant difference in all the dependent variables measured in this experiment can be the short duration of the experiment. The duration of the experiment was 11 weeks, maybe too short for the mycorrhizal fungus to highly colonize the roots. A colonization assessment of the mycorrhizal fungus after 11 weeks recorded a colonization of 30 to 40 %. Nevertheless one can not neglect that there is a difference between the percentage of root colonization and the efficiency of the mycorrhizal strain. Past research indicated that a mycorrhizal infection only could be effective if the colonization exceeded 20 to 30 % (Sanders *et al.*, 1977; Snellgrove *et al.*, 1982). Pinochet *et al.*, (1997) showed a root colonization of 27 % with *Glomus intraradices* being very effective, Cooper & Grandison (1986) observed a total absence of nematodes by a colonization of 10 % with a mix of four AM-fungi, while Saleh & Sikora (1984) needed a colonization of 55 % with *Glomus fasciculatum* to be effective (38 % colonization seemed to be ineffective).
Not only the mycorrhizal strain can make a difference; the environmental conditions can also cause a difference in colonization by the fungus. Jaizme-Vega *et al.* (1991) recorded 38 % of the roots colonized by *Glomus fasiculatum* 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.

These results indicate that the efficiency of a mycorrhizal strain on a given host depends on different 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.

5.1.2.3 The benefits of early mycorrhization

The use of micropropagated plantlets is nowadays established in most of the intensive banana cultivation systems. Micropropagated plantlets are free of diseases, but unfortunately they also lack AM-fungi. Successful inoculation of mycorrhizal fungi at the beginning of the acclimatization period has been demonstrated (Jaizme-Vega *et al.*, 1997; Delvaux *et al.*, 1998). This early mycorrhization is necessary because 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 AM-fungal inoculation may affect the nature of the interaction (Hussey & Roncadori, 1982; Smith, 1987). When plants are inoculated with mycorrhiza several weeks, or even months before being challenged with the pathogen, the mycorrhiza can colonize the root and can grow and develop freely without the influence of the pathogen. Like that, the plant can build up a mechanism for resistance or tolerance against plant-parasitic nematodes. Vaast *et al.* (1998) showed that late mycorrhizal inoculation (added simultaneously with nematodes) did not enhance tolerance of the host plant to nematodes. After 7.5 months, nematodes even decreased mycorrhizal colonization of these late-mycorrhizal plants by half and their biomass was only 20 to 30 % that of the controls.
Although precolonization with AM-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 AM-fungi 2 to 4 months before they are planted into the field (Hussey & Roncadori, 1982; Smith, 1988). This inoculation method was used in many studies (Davis & Menge, 1980; Cooper & Grandison, 1986; Smith *et al.*, 1986b). An established period of three weeks was suggested by Roncadori & Hussey (1984), a period of 6 weeks was used by Elsen (pers. communic.) although Suresh & Bagyaraj (1984) found plants inoculated with mycorrhiza 7 days prior to nematodes had lower nematode populations and high percentage of colonization. The inoculation method used in the experiment performed in this study consisted of an inoculation of the AM-fungi 4 weeks before the inoculation of the nematodes.

5.1.2.4 Difference in pathogenicity between populations of *R. similis*

Many studies performed during the last decades indicated that there is a difference in pathogenicity between different populations of *R. similis* (Sarah *et al.*, 1993; Fallas *et al.*, 1995; Fallas *et al.*, 1996; Hahn *et al.*, 1996). In our experiment, we used a population with high pathogenicity, originating from Ivory Coast, and a population with low pathogenicity, originating from Australia. Where it comes to the number of nematodes, measured per gram of root and the total number of nematodes in the roots, the results of the inoculation with the population originating from Ivory Coast were significantly higher than the results of the inoculation with the population originating from Australia. Also the reproduction rate was significantly higher for the population originating from Ivory Coast.

The parameter 'nematodes per gram of root (= density) however has to be taken with care. When mycorrhizal treatments are compared to non-mycorrhizal treatments, a significantly lower final nematode population can be found in the mycorrhized roots. This can be due to the changes in root morphology of mycorrhized roots such as increase in number of adventitious roots, decrease in their mean length, and greater branching, capable of supporting larger nematode populations. The result is thus a dilution of the nematode root population (Jaizme-Vega *et al.*, 1997). When this is the case, a higher root weight is recorded for the mycorrhized roots. In our experiments however, no difference was found in the root weight between the different treatments, excluding this statement. The significant difference in the number of nematodes per gram of root between the populations originating from Ivory Coast and Australia, and their difference in reproduction rate can thus be attributed to the difference in pathogenicity between these populations.

5.1.2.5 Effect of nematodes on the mycorrhizal colonization

The results found in the literature concerning the effect of nematodes on the mycorrhizal colonization are rather diverse. In several studies nematodes seemed to suppress the colonization by mycorrhiza (Smith *et al.*, 1986b; Umesh *et al.*, 1988; Pinochet *et al.*, 1995; Jaizme-Vega & Pinochet, 1997) while in other studies no effect was recorded (Jaizme-Vega *et al.*, 1997). Many hypotheses exist about the suppressive effect of plant-parasitic nematodes on the colonization of AM-fungi. It is thought that a reduction of space for fungal colonization after several nematodes penetrated the roots, can play a role in the suppressed colonization (Smith, 1988). Also a decrease in supply of carbohydrates

in the roots after nematode penetration is thought to limit fungal colonization (Wallace, 1987).

In the experiment performed in this study, no significant differences could be observed on the percentage of roots colonized with AM-fungi between the different treatments. We can see however that the mycorrhization tended to be higher when the *R. similis* population originating from Ivory Coast was present. We believe that the plant or the mycorrhiza can 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 alter, making the roots maybe more attractive for the mycorrhiza, resulting in an increase in mycorrhizal colonization. The exact mechanisms involved in this interaction are not yet clearly understood and thus need more investigation.

5.1.3 Conclusion

Very low plant height, shoot weight and root weight was recorded. This was probably due to the very low substrate used in the experiment.

No differences in plant growth characteristics, 11 weeks after the inoculation of the mycorrhiza, could be observed, showing that the banana plants probably need more time to optimize the benefits they can take from the symbiosis. Other reasons can be the low mycorrhizal dependency of the cultivar Poyo or the too low colonization of the AM-fungus. More knowledge about the suitable mycorrhiza-host couples is needed to see if the mycorrhizal strain is effective on a given host.

A positive influence of the presence of AM-fungi on the total number of nematodes in the roots was observed. This influence was more obvious regarding the population originating from Ivory Coast.

We could also reconfirm the statement that the population of *R. similis* originating from Ivory Coast is more pathogenic than the population originating from Australia.

No influence of the nematodes was observed on the root colonization by the AM-fungus when the nematodes were inoculated 4 weeks after the inoculation of the mycorrhiza.

This experimental setup in culture chamber conditions is a reproduction of the situation in the field. The conditions are more controlled, making it an appropriate design to study the interaction between host, mycorrhiza and nematodes. Especially for long time-scale experiments, where the reproduction of the nematodes in the mycorrhized or non-mycorrhized roots can be studied and compared, this experimental setup is usually used.

5.2 Comparison of two procedures for the inoculation of mycorrhizal fungi.

The aim of this experiment was to investigate whether there is a difference in plant growth characteristics between plantlets grown on a substrate where the mycorrhizal inoculum was homogenized with the substrate and plantlets grown on a substrate where mycorrhiza were added in a layer, between two layers of substrate. Also the frequency of root colonization by the mycorrhizal fungus was studied.

5.2.1 Results

The growth response of 'Obino l'Ewai' banana to the mycorrhiza *G. mosseae* is listed in Table 5.4. The mycorrhized plants showed a significantly ($P \leq 0.01$) higher plant height compared to the control plants. Moreover, the plants where the mycorrhiza were homogenized with the substrate showed a significantly ($P \leq 0.01$) higher plant height than the plants where the inoculum was added in a layer. For the fresh shoot weight, no significant difference could be observed between the treatment where the inoculum was added in a layer and the control. The treatment 'homogeneously' however showed a significantly ($P \leq 0.05$) higher fresh shoot weight.

Table 5.4: Growth response of 'Obino l'Ewai' banana plantlets to inoculation with *Glomus mosseae*, 9 weeks after inoculation. Inoculum is added to the substrate in two different ways: homogeneously or in a layer.

Treatment	Plant height (cm)	Fresh shoot weight (g)	Fresh root weight (g)	Number of leaves
Homogeneously	9,41 ± 1,66 a	4,89 ± 1,54 a	2,08 ± 0,43 a	7,1 ± 0,90 a
Layer	5,95 ± 1,66 b	2,58 ± 1,17 b	1,27 ± 0,46 b	6,5 ± 0,92 ab
Control	3,01 ± 1,44 c	1,02 ± 0,87 b	0,51 ± 0,39 c	5,5 ± 0,79 b

Data are means of eight replicates for the treatments 'homogeneously' and 'layer' and of six replicates for the treatment 'control'. Means in the same columns followed by the same letter do not differ according to Tukey's test at $P \leq 0.05$. For the factor 'plant height', means followed by a different letter differ according to Tukey's test at $P \leq 0.01$.

Regarding the parameter fresh root weight, the same significant ($P \leq 0.05$) differences could be observed as for the plant height. Mycorrhization enhanced the root system

development of the plants, and this is more expressed in de treatment with the homogeneously added inoculum. The plants grown on a substrate where the inoculum was added homogeneously had a significantly ($P \leq 0.05$) higher number of leaves.

Another aim of the experiment was to study the frequency of root colonization in the primary and secondary roots for the different treatments. These results are given in Table 5.5. Again, significant ($P \leq 0.01$) differences could be observed between the different treatments, but here, the treatment where the inoculum was added in a layer showed a significantly higher root colonization compared to the treatment where the inoculum was added homogeneously. If we compared the different parts of the root, we could conclude that the colonization in the secondary roots is significantly ($P \leq 0.01$) higher than the colonization in the primary roots, and this in both treatments.

Table 5.5: Frequency of root colonization of *Glomus mosseae* in 'Obino l'Ewai' banana roots, 9 weeks after inoculation.

Treatment		Amount of intersections mycorrhizated[z]	% Colonization
Primary roots	Homogeneously	21/1215	1,7 ± 1,9 a
	Layer	175/1009	16,4 ± 6,2 b
Secondary roots	Homogeneously	117/1138	10,2 ± 3,4 b
	Layer	410/936	44,2 ± 7,3 c

Data are means of eight replicates. All percentages were arcsin (x/100) transformed prior to analysis. Means in the same columns followed by the same letter do not differ according to Tukey's test at $P \leq 0.01$.

[z] The number after the slash indicates the amount of intersections on the slide; the number before the slash is the amount of intersections where a mycorrhizal structure could be observed on the intersection.

5.2.2 Discussion

5.2.2.1 Plant growth characteristics

As AM-fungi are obligate symbiotic soil fungi, they need the roots of the host plant to complete their life cycle. There are three growth phases of the AM-fungus prior to colonization of the root during which the host plant may influence the fungus: spore

germination, hyphal growth through the soil, and recognition/appressorium formation. The first phase, the spore germination, is unlikely to be under direct influence of the root. Spores have been germinated in experimental situations in absence of the roots, *in vitro* (Bécard & Fortin, 1988) or in soils (Douds & Schenck, 1991). It was thus unlikely to see any difference in spore germination between the two mycorrhizal treatments of our experiment.

The host root however does influence the growth and morphology of a mycorrhizal hypha as it grows through the soil. The hypha branches profusely in the vicinity of the root. This has been noted *in vitro* using root organ culture (*Agrobacterium rhizogenes* Riker (Ri T-DNA) transformed carrot roots) (Bécard & Fortin, 1988; Douds & Nagahashi, 2000) and also *in situ* (Giovanetti *et al.*, 1993). In an experiment with transformed roots and *G. Gigantea*, Douds & Nagahashi (2000) observed a changed branching pattern, from scattered, long branches in the presence of a dilute signal to tight clusters of profuse branching as the exudate signals become more concentrated. This response could be a strategy, allowing the fungus to efficiently explore the soil for a potential host root exuding the signal from a distance (low concentration) yet branch profusely to find a site for the formation of an appressorium when very close to the root surface (high concentration) (Nagahashi *et al.*, 1998). So in this step, an explanation can be found for the differences in growth response between the two mycorrhizal treatments. In the treatment where the mycorrhiza were added homogeneously, the germinated spores were distributed all over the substrate while in the treatment where the inoculum was added in a layer, the germinated spores were located only in a layer of substrate. The chance of the fungus to find a host root was thus greater with the homogeneously added inoculum.

The next step in the interaction between root and AM-fungus prior to colonization is the formation of the appressorium. In the experiment of Douds & Nagahashi (2000), appressoria were formed on epidermal cell walls within 10 days of growth, but not on cortical or vascular cell walls. They thus conclude that this step is a contact recognition process, not requiring a chemical signal. As the interaction between the fungus and the host root happened earlier in the homogeneous added mycorrhiza, like stated in the previous paragraph, we could thus also conclude that the formation of appressoria on the epidermis and the succesful infection was more likely to happen in the homogeneously treatment.

From these appressoria, inter- and intracellular hyphae colonize the root cortex. Branches from the intercellular hyphae penetrate the cortical parenchyma cells and branch profusely, giving rise to intracellular arbuscules, which are thought to be the main sites of nutrient exchange between the symbionts. The possible earlier development of arbuscules

in the plants where the mycorrhizal inoculum was added homogeneously could thus be the reason for the significantly higher plant height, fresh shoot weight and fresh root weight, compared to the treatment where the mycorrhizal inoculum was added in a layer to the substrate.

The first weeks of the mycorrhizal colonization, the fungus needs a large amount of C to develop. These amounts of C are taken from the host plant, making those nutrients unavailable for the host (Fitter, 1991). This often results in a negative growth response of the host plant. This phenomenon dies out after several weeks. In our experiment, plants were extracted 9 weeks after the inoculation of the fungus. As in the treatment where the mycorrhizal inoculum was added homogeneously, the mycorrhizal infection of the roots by the fungus could start almost directly after the inoculation, it is to believe that the phenomenon of growth reduction of the host plant died out after a few weeks. After this phase, the host plant could benefit directly from the symbiosis, resulting in a better growth response, as could be observed in our experiments. In the treatment where the mycorrhizal inoculum was added in a layer to the substrate, it probably took a longer time period before the fungus interacted with the roots. It is thus possible that the plants of this treatment just left the stage of growth reduction and were only since a short time period taking benefit of the symbiosis. We could see that these plants certainly could benefit from the symbiosis because they had a significantly higher plant height and fresh root weight than the control plants. Also in the fresh shoot weight, there was already a trend for a higher weight compared to the control plants, however, the higher weight was not yet significant.

5.2.2.2 Colonization effectiveness

Significant differences were observed on the root colonization between the primary roots and the secondary roots for both treatments. It is clear that the colonization is higher in secondary roots, as it are the secondary roots who explore the soil volume thus can come in first contact with the hyphae present in the soil. Mycorrhizae also prefer to penetrate in young root tissue, giving another explanation for the higher colonization in the secondary roots.

Both in the primary and the secondary roots, the mycorrhizal infection, measured as the percentage of the root system infected, was significantly higher in the treatment where the mycorrhizal inoculum was added in a layer. The percentage of roots colonized by the mycorrhizal fungus however, is mostly not related to the effectiveness of the symbiosis.

Carroll *et al.* (1985) observed high colonization percentages in the roots of some legumes but the lack of control of the symbiosis resulted in poor yields. Smith *et al.* (1992) also questioned whether a "super-mycorrhizal" plant in which the infection spreads very rapidly and reaches high values under all environmental conditions, would be efficient with respect to P uptake and plant growth.
The arbuscule formation is the most important step in the symbiosis leading to special structures for the exchange of nutrients between both partners. We can thus only guess that in the treatment where the mycorrhizal inoculum is added homogeneously, the arbuscule formation was more expressed than in the treament where the inoculum was added in a layer, resulting in higher plant growth characteristics. It is also believed that the amount of 'branched absorbing structures (BAS)' (Bago *et al.*, 1998) has an influence on the efficiency of the symbiosis. These structures are part of the extraradical mycelium and are believed to be the preferential sites for mineral nutrient acquisition from the soil (see paragraph 2.3.3.1 pg 28).

Also the mycorrhizal dependency of a cultivar can play a role in the percentage of root infection by a AM-fungus that can be obtained in the cultivar. Gerdemann (1975) defined mycorrhizal dependency as "the degree to which a plant relies upon the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility" (see paragraph 5.1.2.1). Nevertheless, the difference in root colonization and the additional difference in growth response in this experiment cannot be attributed to a difference in mycorrhizal dependency because the experiments were performed with banana plants of the same genotype.

5.2.3 Conclusion

The idea to perform this experiment came from the fact that in two known laboratories (CIRAD, Montpellier, France and The Laboratory of Tropical Crop Improvement, K.U.Leuven, Belgium) these different inoculation procedures were used. The results of this experiment however cannot show a preference to one of the two methods. With a duration of the experiment of only 9 weeks, the method of adding the inoculum homogeneously to the substrate seemed to give better results regarding plant growth, compared to the method where the inoculum was added in a layer, surrounded by two layers of substrate. Additional experiments with a longer duration would be necessary to draw any conclusions on the plant growth characteristics after more than 9 weeks of

growth. There is still a possibility that the plants to which the inoculum was added in a layer can show better plant growth characteristics in a longer time experiment because here, the percentage of root infected by the AM-fungus was significantly higher. The factor of interest in this discussion is the intensity of the colonization, and especially the number of arbuscules formed in the roots of the plant in both treatments. This should be a factor to be observed in a following experiment. Also the presence of 'branched absorbing structures' has to be investigated as they play a significant role in the uptake of nutrients from the soil by the mycorrhiza.

We can conclude that the effectiveness of the mycorrhizal strain was high, resulting in significantly higher plant growth characteristics between the mycorrhized plants and the control plants, and that based on this experiment, we cannot show any preference to one of the two inoculum methods used.

5.3 Interaction between mycorrhiza (*Glomus proliferum* and *Glomus* sp.) and *Radopholus similis* on the *Musa* cultivar Poyo.

The goal of this experiment was to investigate the initial attraction and penetration of the burrowing nematode *R. similis* to/into the roots of young banana plantlets inoculated with or without AM-fungi. The duration of the experiment was 24 hours and 48 hours.

5.3.1 Results

In this experiment, performed in pot-culture, two different *R. similis* populations were used: a highly pathogenic population originating from Cameroon and a lowly pathogenic population originating from Australia. The nematodes were inoculated to banana plantlets which were either inoculated with a mycorrhizal fungus (*G. proliferum* or *Glomus* sp.) or left without mycorrhiza. Nematode penetration was investigated in roots mycorrhized with *G. proliferum* 24 hours after inoculation, and in roots mycorrhized with *Glomus.* sp. 24 hours and 48 hours after inoculation.

Since in all the treatments only a low number of nematodes was observed inside the roots, the observation of the experiment was cut short. No observation was made for the treatment *Glomus* sp., 24 hours, and for the other treatments not all the replicates were observed. Table 5.6 gives the results obtained in this experiment. No statistical analysis was performed because of a lack of data.

Table 5.6: Penetration of two different *Radopholus similis* populations (Australia and Cameroon) in banana roots, alone, in combination with *Glomus proliferum* (treatment of 24 hours after inoculation of the nematodes) or in combination with *Glomus* sp. (treatment of 48 hours after the inoculation of the nematodes).

Treatment		24h/200 nem	48h/400 nem
- AM-fungi	Australia	7 ± 7	40 [z]
	Cameroon	27 ± 30	32 ± 20
+ AM-fungi	Australia	6 ± 10	33 ± 23
	Cameroon	3 [z]	23 ± 16

Data are means of one to five replicates.

[z] Data consisting of only one replicate; no standard deviation could be measured.

Plants inoculated with *G. proliferum* showed no mycorrhizal colonization while the inoculum of *Glomus* sp. was more effective, giving a colonization of 34.75 % (Table 5.7). This colonization consisted of a network of hyphae but almost no vesicles were observed.

An additional test was performed in order to estimate the colonization of *G. 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 47.3 %. Almost half of the mycorrhizal structures observed were vesicles. Table 5.7 gives an overview of the colonization of the three mycorrhiza species.

Table 5.7: Colonization of three mycorrhiza species in the roots of banana plantlets, 8 weeks (*Glomus intraradices*), 9 weeks (*Glomus* sp.) and 10 weeks (*Glomus proliferum*) after inoculation.

Treatment	Amount of intersections mycorrhizated[z]	% Colonization
Glomus proliferum	0	0
Glomus sp.	116/334	34,8 ± 9,0
Glomus intraradices	249/525	47,3 ± 12,4

Data are means of five (*Glomus proliferum* and *Glomus* sp.) or six (*Glomus intraradices*) replicates.

[z] The number after the slash indicates the amount of intersections on the slide; the number before the slash is the amount of intersections where a mycorrhizal structure could be observed.

5.3.2 Discussion

5.3.2.1 Nematode penetration into the roots

The initial attraction and penetration of the burrowing nematode *R. similis* to/into the roots of banana plantlets was studied in culture chamber conditions. The results showed that the experimental setup in these conditions was not appropriate for this study. Almost no nematodes were found in the roots after 24 or 48 hours, indicating that the time between inoculation and observation was probably too short, that the volume of the pot (250 ml) was probably too big, or that the quantity of inoculum (200 or 400 nematodes) was too little. One can conclude that, to study the initial attraction and penetration of nematodes to/into roots, a longer time interval between inoculation and observation is needed. Another possibility can be the development of another experimental setup (see below, paragraph 5.4).

5.3.2.2 Mycorrhizal colonization

One of the mycorrhizal species used in this experiment was *G. proliferum*. This newly 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 as no colonization was observed. In the same conditions, *Glomus* sp. was able to colonize the

roots of the banana plantlets: a colonization of 34.8 % was found after 9 weeks. The frequency of colonization by the mycorrizal strain *G. intraradices*, measured in an additional experiment, but in almost the same environmental conditions, gave also good results: a colonization of 47.3 % was observed after 8 weeks.

The mycorrhizal structures formed by *Glomus* sp. showed a total different morphology compared to the structures formed by *G. intraradices*. With *Glomus* sp. a network of thick hyphae was found and almost no vesicles were observed (Figure 5.1). Colonization with *G. intraradices* in the same conditions consisted of many vesicles and a really thin network of hyphae (Figure 5.2). This confirmed the results obtained by Pinochet *et al.* (1995), who also observed many vesicles in experiments where *G. 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.

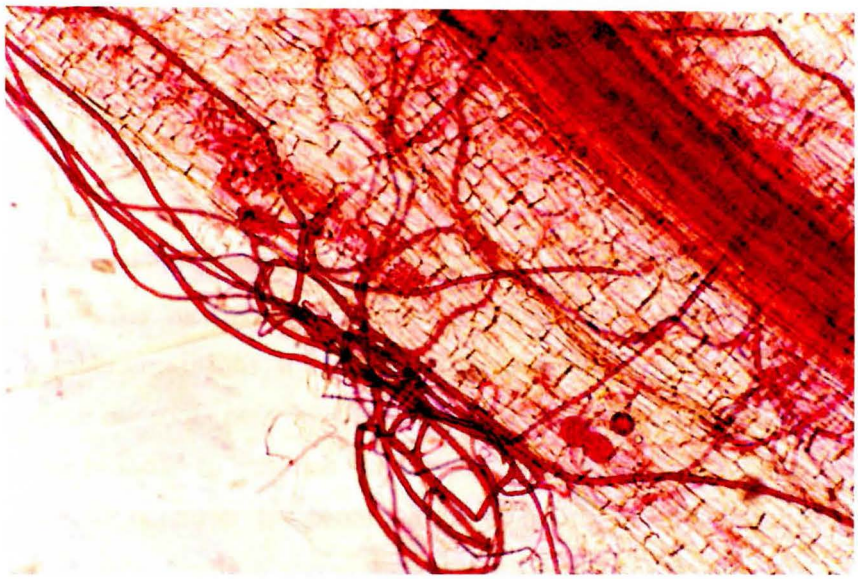


Figure 5.1: Hyphae and some vesicles observed in banana roots colonized by *Glomus* sp. The roots were colored by fuchsine acid and observed at a 40 x magnification.

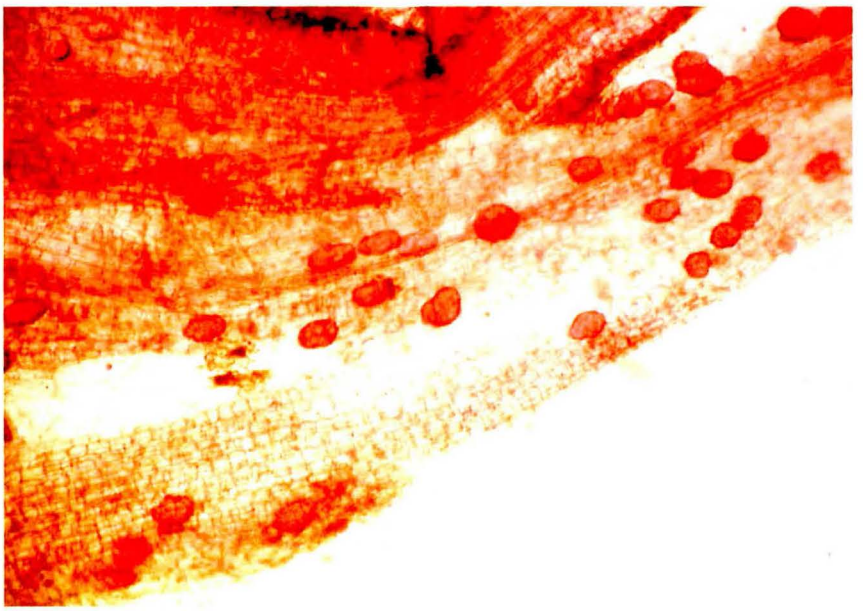


Figure 5.2: Vesicles observed in roots colonized with *Glomus intraradices*, colored by fuchsine acid and observed at a 40 x magnification.

5.3.3 Conclusion

As almost no nematodes penetrated the roots of the banana plantlets, 24 hours and 48 hours after inoculation, we can conclude that with this experimental setup in culture chamber conditions, it is not possible to study the attraction and penetration of nematodes to/into the roots only 24 hours and 48 hours after inoculation. The volume of the pot has to be reduced to enhance the chance that nematodes can find their way to the roots and thus can penetrate the roots within 24 hours. Another possibility is to increase the time between inoculation and observation. Because of the low penetration of the nematodes in the roots, it was not possible to draw any conclusions about a possible interaction between the mycorrhizal fungus and the nematodes. There is also a possibility that the mycorrhizal colonization was too low to be highly effective. This low colonization could be due to the conditions in which the experiment is performed, or to the host-mycorrhiza relationship. The development of the best host-mycorrhiza couples is currently under study.

Part II: Experiments performed in Petri dishes

5.4 Initial attraction and penetration of *Radopholus similis* to/into banana plantlet roots.

The aim of this experiment, performed in Petri dish, was to have a first approach on the mechanisms involved in the attraction and penetration of nematodes into the roots of banana plantlets. For this, a small scale experiment was developed. 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 was performed to investigate whether the physiology of the root changed after the root was cut from the plant.

5.4.1 Results

Almost no nematodes penetrated the root 24 hours after inoculation, except for the inoculation procedure 200/200 nematodes (Table 5.8). The results show an increase of nematodes penetrating the roots when the time between inoculation and observation was greater. At 72 hours, almost no differences could be observed between the different inoculation procedures.

Table 5.8: Penetration of *Radopholus similis* (Cameroon 12) into the roots of banana in Petri dish, 24 hours, 48 hours and 72 hours after inoculation with 200 or 400 nematodes.

time before observation	nematodes inoculated		
	200 nem[a]	400 nem[a]	200/200 nem[z]
24 h	6 ± 11 ab	8 ± 7 bc	66 ± 72 cd
48 h	1 ± 1 a	38 ± 32 bcd	74 ± 93 cd
72 h	66 ± 64 cd	69 ± 54 cd	83 ± 34 d

Data are means of five replicates. All nematode densities were $\log_{10}(x+1)$ transformed prior to analysis. Means in the table followed by the same letter do not differ according to Tukey's test at $P \leq 0.05$ (two-way ANOVA).

[a] 200 respectively 400 nematodes were inoculated on the surface of the sand, along the root.

[z] Two curves of 3 mm deep were made on both sides along the root at a distance of 2 mm from the root. The inoculum (two times 200 nematodes) was inserted in these curves.

The next parameter to be investigated was whether there was a difference in the fragment of the root used in the experiment, to obtain a maximum penetration of the nematodes. Root fragments including meristem were compared to root fragments without meristem. No significant differences could be found between the two treatments, but there is a trend that nematodes prefer to enter close to the meristem (115 out of 400 nematodes penetrated, compared to 24 in the roots without meristem) (Table 5.9) even if no significant difference could be observed. This is due of the high variability in the data (in accordance with the high standard deviation, Table 5.9).

The quantity of water that had to be added to the sand to obtain an optimal activity of the nematodes and thus a possible better penetration into the roots was investigated. When the sand was saturated with water, the nematode penetration tended to be higher than when the sand was not saturated (60 out of 400 nematodes, compared to 32, Table 5.9), but this difference was not significant.

In another part of the experiment, the Petri dish, containing the root embedded in sand, was placed in a box at 27 °C for 24 hours before being inoculated with nematodes. We assumed that there could be an alteration in root exudates released by the roots after they had been cut. The aim of this study was to investigate whether this alteration of root exudates had an effect on the penetration of the nematodes. In the case of the inoculation 24 hours after the root had been cut, significantly ($P \leq 0.001$) less nematodes penetrated the roots. Only 12 of the inoculated nematodes (400 nematodes) penetrated the root,

compared to 131 that penetrated the root when they were inoculated right after the root had been cut (Table 5.9).

Table 5.9: Penetration of *Radopholus similis* (Ivory Coast) into the roots of banana plantlets in Petri dish. The inoculation quantitiy was 200/200 nematodes[z]. The different parameters tested were: the segment of the root (meristem included (meristem +) or meristem not included (meristem -)), the humidity of the sand (5.5 ml or 6 ml of water added to the sand) and the time between cutting of the root and inoculation of the nematodes (storage 0 h or 24 h).

	Treatment	Time between inoculation and observation	
		72 h	48 h
A	meristem -	24 ± 27 a	~
	meristem +	115 ± 82 a	~
B	humidity 5,5 ml	32 ± 27 a	~
	humidity 6 ml	60 ± 57 a	~
C	storage 0 hours	~	131 ± 77 a
	storage 24 hours	~	12 ± 16 b

[z] Two curves of 3 mm deep were made on both sides along the root at a distance of 2 mm from the root. The inoculum (200/200 nematodes) was inserted in these curves.

Data are means of eight replicates. All nematode densities were $\log_{10}(x+1)$ transformed prior to analysis. Means of the same experiment (A, B or C), followed by the same letter do not differ according to Tukey's test at $P \leq 0.05$. For the treatment 'adaptation', means followed by a different letter differ according to Tukey's test at $P \leq 0.001$.

~ No experiments were performed for this combination treatment-observation time.

Different places along the root to add the inoculum were investigated. When the inoculum was added in the middle of the root segment, 72 nematodes out of 400 penetrated the root. Out of these 72 nematodes, 36 nematodes penetrated the root on the place where the root had been cut. When the inoculum was added on the meristem, opposite to where the root had been cut, 78 nematodes (out of 400) penetrated the root. Out of these, 27 nematodes found their way to the cut and entered there (Table 5.10).

Table 5.10: Penetration of *Radopholus similis* (Ivory Coast) into the roots of banana plantlets in Petri dish. 400 nematodes were added in the middle of the root (Inoculation middle) or on the meristem (Inoculation meristem). The time between inoculation and observation was 72 hours.

Treatment	Total number of nematodes penetrated in the root	Number of nematodes penetrated in the cut
Inoculation middle	72 ± 50 a	36 ± 26 a
Inoculation meristem	78 ± 36 a	27 ± 27 a

Data are means of eight replicates. All nematode densities were $\log_{10}(x+1)$ transformed prior to analysis. Means in the same columns followed by the same letter do not differ according to Tukey's test at $P \leq 0.05$.

5.4.2 Discussion

To study the initial attraction to and penetration into the roots of banana plantlets, a new experimental setup was developed. Experiments were performed in Petri dishes, in order to have 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 to the more realistic pot-experiments, but it could be useful to study the penetration of nematodes into the roots of banana plantlets, 24 hours, 48 hours or 72 hours after the inoculation of the nematodes.

The inoculation procedure where two times 200 nematodes were added to the sand seemed to give the best penetration of the nematodes into the roots. In this inoculation procedure, 2 curves were made in the sand on both sides along the root and the inoculum was added in these curves. This was designed to prevent the inoculum to spread out, contrarily to what happened in the other inoculation procedures. The nematodes thus stayed closer to the root after inoculation, and so, they could find their way to the root more easily. When the inoculum was added on top of the root, the inoculum spread out. The nematodes were thus further removed from the root, so it took longer to reach the root and to penetrate. This was probably the reason why there was a difference in penetration of the nematodes between the three inoculation procedures, 24 hours and 48 hours after inoculation, but why this difference died out, 72 hours after inoculation.

Harley & Smith (1983) postulated that nematodes prefer to enter at or near the root tip. Also Zhao *et al.* (2000) stated that the infection of most root-associated nematodes is initiated in newly generated elongating tissue several millimeters behind the root apex, and thus does not occur in mature tissues. This statement was investigated by comparing the penetration of nematodes in two different segments of the root, one including the meristem, the other without meristem. Results obtained in this experiment confirmed these statements. Much more nematodes penetrated into the root fragment including the meristem compared to the root fragment excluding the meristem.

The movement of the nematodes was facilitated when the sand was saturated with water. Nematodes are used to live in aquatic conditions so a soil saturated with water gives them the opportunity to move freely, compared to a more dry soil. When the sand was drier, less nematodes penetrated the root. The surface force on dry sand did not allow nematodes to move freely.

In all those experiments, 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 added either 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 (Figure 5.3). This indicates that this setup is artificial and has to be improved to make it useful for further experiments.

In a last experiment, the physiological change of the root after it had been cut was investigated. When the root in the Petri dish was placed in a box at 27 °C for 24 hours, the physiological state of the root was altered. Nematodes inoculated to this root had a significantly lower penetration compared to the penetration of the nematodes that were inoculated directly after the root was cut. These results confirm the statement that root exudates are altered when a root is damaged, physically or in response to nematode invasion (Binks *et al.*, 1997). This is a second proof that the artificial setup in this condition does not resemble the real physiological state of the root.

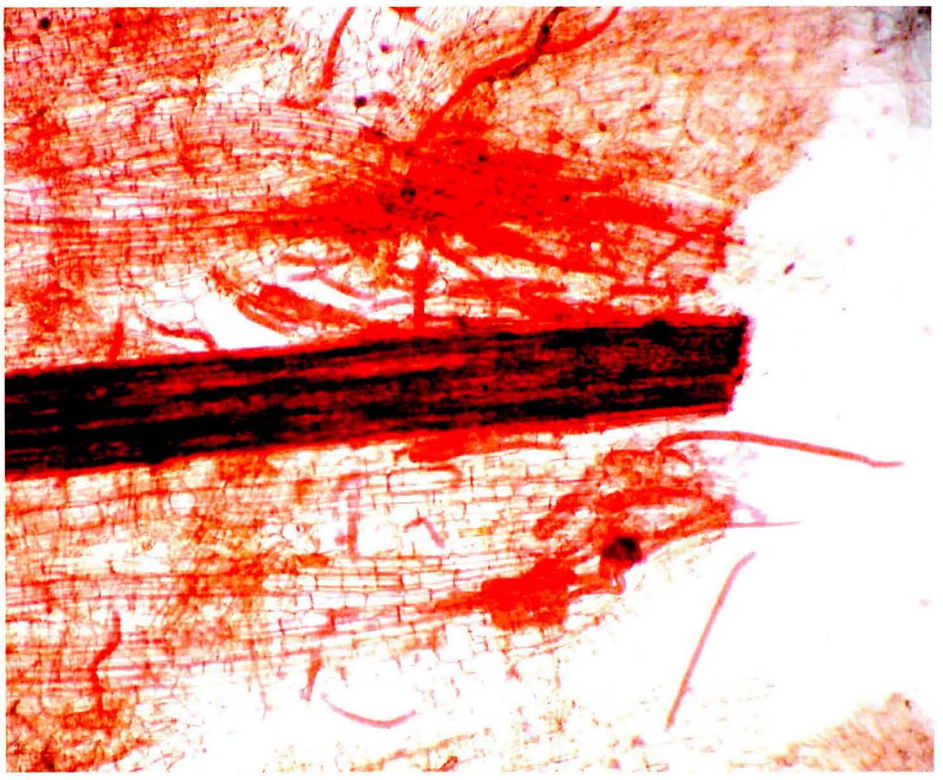


Figure 5.3: A root segment of 4.5 cm, including the meristem, was placed in a Petri dish (∅ 5.5 cm), filled with 30 g of sand and 6 ml of water. 400 nematodes (*Radopholus similis*, population Ivory Coast) were inoculated on the meristem. 34 % of the nematodes that penetrated into the root found the way to the place where the root was cut and penetrated in the cut.
The roots were colored by fuchsine acid and observed at a 40 x magnification.

5.4.3. Conclusion

To study the initial attraction to and the penetration into the roots of banana plantlets, more controlled conditions were needed. Therefore, a small scale experiment was developed. In this experiment, we tried to investigate some factors to optimize the experimental setup in Petri dish conditions. With this setup, the behavior of the nematodes, directly after the inoculation could have been studied. The next step would have been to study this initial attraction to and penetration into the roots, in presence or absence of mycorrhizal fungi. This experimental setup would have been useful to study the interaction between mycorrhiza and nematodes on a host plant, directly after the inoculation of the nematodes.

The setup, however, had some shortcomings, making it not appropriate. The diameter of the Petri dishes was only 5.5 cm, making it necessary to use excised roots. This physical damage altered the physiological status of the root causing an alteration in the behavior of the nematodes.

5.5 Initial attraction of different *Radopholus similis* populations to different banana cultivars *in vitro*.

The aim of this experiment was to study the initial attraction of two *R. similis* populations with a difference in pathogenicity, to two different *Musa* cultivars with a difference in resistance/susceptibility to nematodes. The experiment was performed *in vitro* in Petri dishes (∅ 13.5 cm).

5.5.1 Results

Apparently no differences could be observed between the attraction of the two different nematode populations to the roots of the banana plants (Table 5.11). On one hand, there seemed to be no difference between the two nematode populations in how they were attracted to the roots, and on the other hand, no difference could be observed between the two banana cultivars in how they attracted nematodes.

Also no correlation could be found between the time between inoculation and observation, and the penetration of the nematodes. In the treatment where Yangambi km 5 was the host plant, it seemed that more nematodes penetrated the roots after 3 hours compared to the nematode penetration after 2 hours but this can just be coincidence because the opposite could be observed in the experiments performed with Obino l'Ewai: more nematodes penetrated the roots 2 hours after inoculation compared to 3 hours after inoculation.

Several nematodes fell into irregularities in the medium during their movement, and some other nematodes could not be recovered from the medium at time of observation. Nematodes were also observed to make a long track without finding their way to the root. For the nematodes who generally moved in the direction of the roots, no differences could be observed between the different populations on the track they made during the 2 or 3 hours of movement. In all the treatments, all the nematodes had a looping/spiralling behaviour and almost no direct movement to the root could be observed.

Table 5.11: Behavior of two different *Radopholus similis* populations in a Petri dish filled with MSR (Modified Stullu Romand) medium and containing a resistant (Yangambi km 5) or susceptible (Obino l'Ewai) banana plantlet (see Figure 4.1 pg 46), 2 or 3 hours after inoculation of the nematodes.

Treatment			penetrated in root	in medium (stop)	in medium (irregularity)	not recovered
Yangambi km 5	Indonesia	2 h	1	15	2	7
		3 h	10	9	1	5
	Uganda	2 h	5	17	1	2
		3 h	12	11	0	2
Obino l'Ewai	Indonesia	2 h	12	11	1	1
		3 h	11	11	2	1
	Uganda	2 h	4	16	3	2
		3 h	2	15	6	2

Figure 5.4: Looping/spiralling behaviour of a nematode observed on MSR medium in a Petri dish containing a banana plantlet (not on picture). The plate is developed following the procedure described in paragraph 4.4.5 pg 59).

5.5.2 Discussion

5.5.2.1 Shortcomings of the experimental setup

Petri dishes are used in experiments when more controlled conditions are desired. This experimental setup facilitates studies on small scale (time and space), compared to greenhouse and/or field studies. The experimental setup used in this experiment is already an improvement of the setup used in experiment 4, because a whole plant can be placed in the Petri dish. However, it still has some shortcomings. (i) While the plants were put on, and a bit in the medium, the medium was damaged, causing irregularities. These acted as a barrier for the nematodes. Some nematodes fell into these irregularities, making it impossible to continue their way to the root. (ii) Only a few nematodes could penetrate the roots, while a lot of nematodes did not find their way to the roots yet, making the time between inoculation and observation too short. Between time intervals of 2 hours and 3 hours, apparently no difference could be observed in the penetration of the nematodes, concluding that a longer time-interval is needed to reach an optimal penetration. (iii) During the observation, a lot of nematodes could not be recovered in the Petri dish. Per Petri dish, five nematodes were inoculated, while sometimes only three or four nematodes were recovered afterwards. The nematodes were inoculated on the medium surface as far as possible from the roots, i.e. close to the wall of the Petri dish. It is now possible that some nematodes directly moved to the wall of the Petri dish, where the medium connects with the dish. Here, they are impossible to find for the observer. This problem can be avoided firstly by inoculating the nematodes not too close to the wall so they can find their way to the root more easily and secondly by marking the spot where the nematodes had been inoculated so it is easier for the observer to find them back. If the observation is started on the spot where the nematode was inoculated, an obvious track can be followed, making it easy to follow the track of the nematode. (iv) It was tried to inoculate the nematodes all approximately on the same distance from the roots. However, this is rather difficult to perform in a round Petri dish in which the roots were placed randomly. Some nematodes were thus inoculated closer to the roots compared to others, enlarging the chance that they penetrate into a root.

To overcome the shortcomings described in (ii) and (iv), a new experimental setup can be proposed (Figure 5.5). In this setup, a plant, rooted for approximately one week has to be placed in a rectangular or square Petri dish, containing medium. Two compartments of the Petri dish have to be separated by a 30 μm membrane, making it impossible for the

roots to grow into the other part, but allowing the nematodes to move to the other compartment. The plant has to grow into the medium for a few weeks until the roots reach the membrane. Five nematodes than have to be inoculated in the other compartment, on the surface of the medium, all on the same distance from the membrane. With this setup, the medium would not be damaged and the nematodes can all be inoculated on the same distance from the roots.

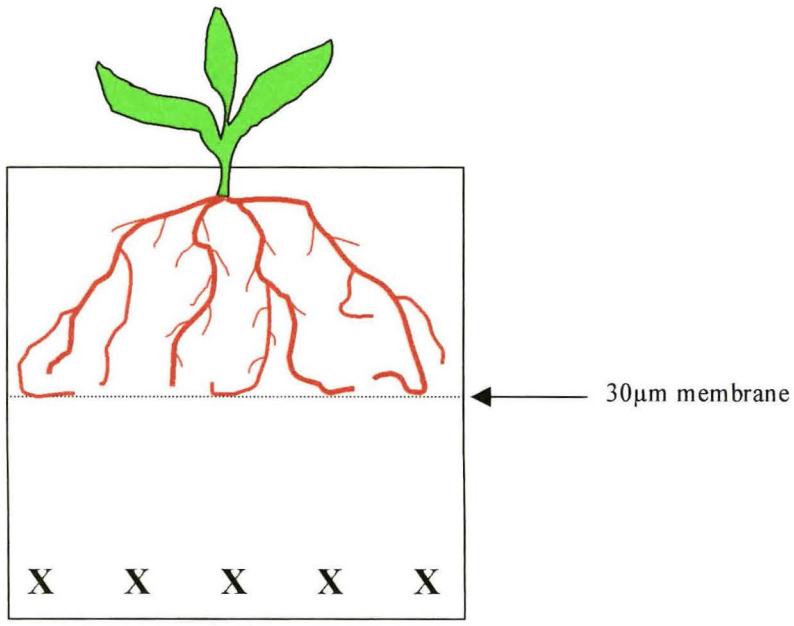


Figure 5.5: Proposed experimental setup to overcome some shortcomings of the setup used in experiment 5. The plant, rooted for one week in a tube, is brought into the Petri dish and has to continue growing in the dish for 4 weeks. Thereafter, nematodes (X) are inoculated on the surface of the medium, all on the same distance from the roots.

5.5.2.2 Pathogenicity of *R. similis* populations

Population biology studies involving *R. similis* populations from the major banana growing areas of the world have demonstrated a large variability in pathogenicity to banana plants (Sarah *et al.*, 1993; Fallas *et al.*, 1995; Hahn *et al.*, 1996; see paragraph, 2.2.2.3). According to Bos & Parlevliet (1995), pathogenicity on a given host is the consequence of two independent parameters (Figure 5.6): aggressiveness (degree of attack or invasion) and virulence (intensity of the symptoms). Aggressiveness being directly dependent on the reproductive fitness of the pathogen (comparative

multiplication rate of different isolates on a given host) and the ability to penetrate or infect.

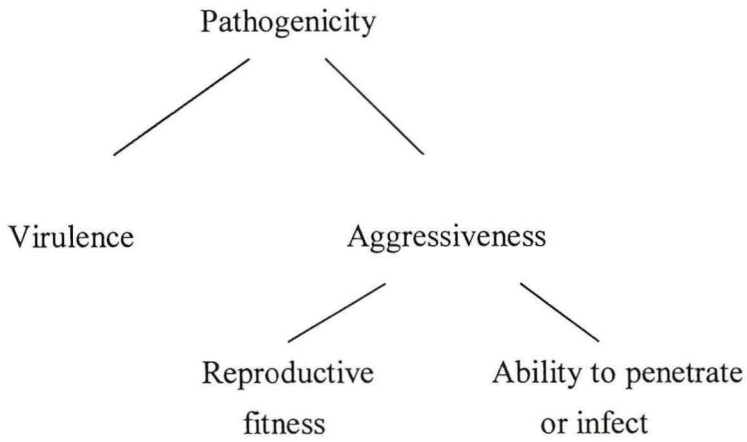


Figure 5.6: Overview of the different parameters involved in the pathogenicity of a parasite (according to Bos & Parlevliet, 1995).

As no differences were observed in the attraction to the roots between the two different nematode populations, it can be assumed that the stage of attraction and penetration does not play a role in the pathogenicity status of a population.

Fallas & Sarah (1995) investigated the damage a population can cause (i.e. pathogenicity), in relation to the density of the pathogen. Between these two parameters, they found a very high correlation (r^2 = 0.960) for the eight nematode populations investigated. They showed that the pathogenicity of the different *R. similis* isolates on banana plants tested in their experiment appeared to be a direct consequence of reproductive fitness (see graph, Annex 6). Because of the high correlation obtained between the reproductive fitness and the induced damaged (pathogenicity) (Fallas & Sarah, 1995), it is, regarding figure 5.6, to accept that the parameters virulence and ability to penetrate or infect do not interact in the system, or interact to give a zero-effect. As the high correlation was obtained for eight different *R. similis* populations, it can be assumed that the two parameters, virulence and ability to penetrate or infect do not interact in the system, so do not influence the pathogenicity status of a nematode population. It can therefore be proposed to use the reproductive fitness as a direct assessment of the pathogenicity of a population.

If the two populations, used in the experiment only differ in reproductive fitness, the difference in damage these populations can induce to their hosts can only be seen after the nematodes have penetrated the roots and reproduced.

5.5.2.3 Resistance/susceptibility of the cultivars

Two different cultivars were tested in the experiment: the partially resistant cultivar 'Yangambi km 5' and the susceptible cultivar 'Obino l'Ewai' (see paragraph 2.2.2.3 and 2.2.2.4). We use the term partially resistant because absolute resistance is very rare in *Musa* (De Waele & Elsen, 2002). As no trend was observed that the nematodes would be more attracted to the susceptible cultivar or to the resistant one, two hypotheses can be stated: (i) the resistance mechanisms (for example the build-up of phenolic cells) were not yet totally developed in the *in vitro* propagated plantlets or (ii) the resistance mechanisms are only active from the moment the nematodes penetrate the roots of the plant.

The plants used in our experiment had rooted on MS-rooting medium for 4 weeks prior to inoculation. In the experiments for early screening for resistance, however, plants were at least 8 to 10 weeks before inoculation (Stoffelen, 2000) or the experiment was started with plants of 4 weeks but the duration of the experiment was at least 4 more weeks (Elsen *et al.*, accepted). This time period is probably needed for the *in vitro* propagated plantlets to develop their resistance mechanism. For Pisang Yari Buaya (PJB), the resistance was confirmed in an *in vitro* experiment, 8 weeks after inoculation (Stoffelen, 2000). For the cultivar Yangambi km 5, however, the partial resistance or resistance, as described in many experiments (Wehunt *et al.*, 1978; Sarah *et al.*, 1992; Price *et al.*, 1994; Fogain & Gowen, 1997) was not confirmed under *in vitro* conditions (Stoffelen, 2000; Orayaj, 2001). It can be assumed that in the early vegetative growth phase, there is a chance that not all the types of resistance are yet expressed. That is probably why no difference could be observed between the attraction of the nematodes to the two different banana cultivars.

Plants synthetize a wide variety of chemical compounds after infection by nematodes (Collingborn *et al.*, 2000). Only a few *Musa* cultivars have so far been investigated for their biochemical reactions to nematodes. Valette *et al.* (1997) found post-infectional production of phenols in the roots of Yangambi km 5 but not in the susceptible Poyo. They also observed a more extended accumulation of flavonoids in the resistant cultivar

after infection. This was hypothesized to contribute to the plant defence strategy, limiting nematode ingress within root tissues (Valette *et al.*, 1996). Binks *et al.* (1997) noticed a production of phytoalexines in the roots of several plants in response to nematode invasion. All these mechanisms are post-infectional, so they are not within the reach of the experiment performed in this study because the aim of this experiment was to investigate whether there is a difference in attraction for the nematodes to a resistant and a susceptible banana cultivar. Because of the short duration of the expiriment, it is hard to believe that the penetration of one nematode can bring about these post-infectional changes to the roots of the host, causing an alteration in the physiology of the root, and effecting the attraction of the four other nematodes in the experiment.
Also the differences based upon constitutive characteristics of the root tissue like lignification in the case of the resistant cultivar Pisang Jari Buaya or suberization in the case of Yangambi km 5 can not provoke a different attraction to the roots of the two cultivars. The lignification and suberization of some root cells can only be a defence mechanism on the moment that nematodes try to penetrate the root.

Root exudates need around 9 hours to diffuse in agar based medium (Klink *et al.*, 1970). In our experiment, the Petri dishes with the plant were kept for around 24 hours before inoculation with the nematodes. Root exudates could thus diffuse throughout the medium in the Petri dish. Anderson *et al.* (1997) could observe that the looping/spiralling behaviour of the nematodes in absence of an attractant or repellent changed into a more straight movement if an attractant or repellent was present. As no difference in the track of the nematodes could be observed, it can be assumed that the root exudates of the two different cultivars did not have a different effect (attraction or repulsion) on the nematodes.

5.5.3 Conclusion

The reason that no difference in nematode attraction to the plants could be observed between the resistant and the susceptible cultivar, can be explained by the fact that the mechanisms for nematode control in resistant cultivars are in general post-infectional.
It is also possible that the mechanisms for resistance in the cultivar Yangambi km 5, like the build-up of phenolic cells or the presence of suberization of some root cells, were not yet expressed in the *in vitro* propagated plantlets.

It was also impossible to detect any differences in attraction to the roots between the two different nematode populations. It therefore can be assumed that the stage of attraction and penetration does not play a role in the pathogenicity status of a population. Fallas & Sarah (1995) found a high correlation between the reproductive fitness and the pathogenicity of eight *R. similis* populations (Annex 6), resulting in an equality in virulence and ability to penetrate or infect between these populations (Figure 5.6 pg 91).

5.5.4 Future prospects

5.5.4.1 Beneficial effects of *in vitro* mycorrhization

Recently, *in vitro* micropropagated plantlets have replaced suckers as a source of planting material for the initiation of new, or the replacement of existing banana fields in the big plantations. The vegetative micropropagation is promising because it produces genetically homogeneous plants in a good phytosanitary condition, and large numbers of plants can be produced all year round (Yano-Melo *et al.*, 1999). Another advantage of micropropagation is that micropropagated plantlets are free of diseases. They however lack AM-fungi. Successful inoculation of AM-fungi at the beginning of the acclimatization period has already been demonstrated by many researchers (Jaizme-Vega *et al.*, 1997; Delvaux *et al.*, 1998) improving the nutrient status of the plants, resulting in a better adaptation to the field conditions. The transfer of the plants from the *in vitro* conditions to the greenhouse is one of the most important steps in the structural and physiological adaptation of the plantlets. It should thus be an even better solution if the plantlets could already be inoculated with the AM-fungi before the acclimatization phase, this means in the *in vitro* stage. As a first step in this objective, the cultivation of several *Glomus* strains on *Agrobacterium rhizogenes* Riker (Ri T-DNA) transformed carrot roots, has already been carried out with success (Fortin *et al.*, 2002).

The next idea would be to interact AM-fungi with *in vitro* rooted plantlets in *in vitro* conditions. Some research has been already performed on this subject (Declerck, pers. communic.) but there is still a long way to go before plantlets, *in vitro* inoculated with AM-fungi, can be transplanted into the field to start a new banana production.

5.5.4.2 Study of mycorrhiza-nematode interactions in *in vitro* conditions

The *in vitro* interaction between plant-parasitic nematodes and AM-fungi on a host has also been studied recently. On this subject, promising results are already obtained on root-organ culture. Elsen *et al.* (2000) could confirm the beneficial effect of AM-fungi on Ri T-DNA transformed carrot roots, inoculated with plant-parasitic nematodes. Root-organ cultures, however, have the disadvantage that they do not represent a whole plant system as the aerial part is not present. As a result, photosynthesis and nutrient flow from the roots to the shoot are absent and the hormone balance is distorted. This root-organ culture has however often been used to study nematode reproduction in presence and absence of AM-fungi (Fortin *et al.*, 2002).

A further step in these experiments would be the study of the interaction of AM-fungi and plant-parasitic nematodes *in vitro* on rooted plantlets. This setup would be less artificial than the root-organ culture so it can be a promising experimental setup to study nematode-mycorrhiza interactions in *in vitro* conditions. Before to arrive on that stage, more research has to be performed on the interaction between AM-fungi and host plant roots *in vitro*.

6 Conclusion

A first experiment was performed on the effect of AM-fungi on the penetration and reproduction of different *Radopholus similis* populations in the roots of banana plantlets. In this experiment, a positive effect of the presence of AM-fungi could be observed. No differences in plant growth characteristics could be detected, but a significantly lower number of nematodes could be recovered from the mycorrhized roots. This effect was more pronounced regarding the population originating from Ivory Coast. The difference in pathogenicity between the two populations used in the experiment (Ivory Coast and Australia) could also be confirmed. The nematodes seemed to have no effect on the root colonization by the AM-fungus when the nematodes were inoculated 4 weeks after the inoculation of the mycorrhiza.

In an additional experiment, two inoculation procedures of AM-fungi were investigated. A better growth response was observed in the procedure where the inoculum was added homogeneously, 9 weeks after the inoculation of the fungus. However, a higher root colonization by the AM-fungus could be assessed in the procedure where the inoculum was added in a layer to the substrate. Therefore, we can assume that the development of arbuscules, the main site for the exchange of nutrients between both partners in the symbiosis and/or the formation of 'branched absorbing structures (BAS)', the main site of nutrient uptake from the soil by the mycorrhiza, were more pronounced in the roots where the mycorrhiza were added homogeneously. To confirm this statement, it would have been necessary to investigate the arbuscule formation in the root systems of both treatments and if possible, the formation of BAS in the substrate. No predictions could be made about the plant growth characteristics and the mycorrhizal root colonization between both treatments on a larger time scale so no conclusions could be drawn about which inoculation procedure would be the most efficient for the host plant.

The experimental setup in culture chamber conditions was shown to be appropriate to study the reproduction of nematodes in the roots. In the next experiment, this setup was used to study only the attraction and penetration, directly after inoculation of the nematodes. The results indicated that for this aim, the time between inoculation and observation was too short.

Another experimental setup was developed to study the attraction and penetration of nematodes in the roots. Experiments were conducted in more controlled conditions (Petri dishes).

In the first Petri dish experiment, only a part of the root was investigated. The physical damage due to the excision of the root changed the physiological status of the root,

resulting in deceiving results. The nematodes had the tendency to penetrate the root on the place where the root had been cut, even when they were inoculated on the opposite part of the root segment.

To overcome this problem, larger Petri dishes were used, to place the whole plant, without damaging any part of the root system. This experimental setup was more appropriate to study the attraction of the nematodes to the roots. The attraction was investigated between two nematode populations with a difference in pathogenicity and two banana cultivars with a difference in resistance to nematodes. No differences could be observed on the attraction of the nematodes to the roots between the different treatments. This is probably due to the equal ability to penetrate the roots of two nematode populations who differ in pathogenicity and the fact that most of the resistance mechanisms in plants are post-infectional, so these mechanisms are not yet expressed at the stage of attraction. Moreover, the resistance of the partially resistant cultivar Yangambi km 5, used as the resistant cultivar in this study, had not yet been confirmed *in vitro*, giving a second explanation why no difference in attraction of the nematodes to the two different cultivars could be observed.

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Annexes

Annex 1: 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 2: Murashige & Skoog (MS) rooting medium for *in vitro* tissue culturing of bananas.

- Fill a beaker with distilled water
- Start heating and stirring
- Add MS powder: 2,17 g/l modified MS powder (basalt salt mixture, with FeNaEDTA at 50% concentration)
- Add 1 ml/l vitamines

  Composition: - glycine: 0,20 g/100 ml
  - thiamine hydrochloride: 0,01 g/100 ml
  - pyridoxine hydrochloride: 0,05 g/100 ml
  - nicotinic acid : 0,05 g/100 ml
- Add 1 ml/l ascorbic acid
- Add distilled water to a volume of 1 liter
- Add a mixture of 30 g/l sugar (sucrose) and 2g/l gelrite
- Adjust the pH to 6,12 at 60 °C
- Boil the medium
- Add the medium to culture vessels: 20 ml in test tube
- Autoclave the test tubes with the medium

Annex 3: White's medium for the production of callus.

Composition of the solutions:

Chemical	Formula	Amount	Solvent	Quantity
Solution 1				
Calcium nitrate	$Ca(NO_3).4H_2O$	3.00 g		
Potassium nitrate	KNO_3	0.80 g	250-ml H_2O	250-ml
Potassium chloride	KCl	0.65 g		
Magnesium sulfate	$MgSO_4.7H_2O$	7.50 g	250-ml H_2O	250-ml
Sodium sulfate	Na_2SO_4	2.00 g		
Sodium phosphate (monobasic)	$NaH_2PO_4.H_2O$	0.19 g	250-ml H_2O	250-ml
Manganous sulfate	$MnSO_4.4H_2O$	0.20 g		
Zinc sulfate	$ZnSO_4.7H_2O$	0.12 g	1000-ml H_2O	250-ml
Boric acid	H_3BO_3	0.06 g		
Potassium iodide	KI	0.03 g		
Cupric sulfate	$CuSO_4.5H_2O$	0.40 g	1000-ml H_2O	1-ml
Molybdenum trioxide	MoO_3	0.04 g		
Solution 2				
Glycine	CH_2NH_2COOH	1.50 g		
Nicotine acid	N:CHC(COOH):CHCH:CH	0.25 g	500-ml H_2O	1-ml
Thiamine	$C_{12}H_{18}C_{12}N_4OS$	0.05 g		
Pyridoxine	$C_8H_{12}ClNO_3$	0.05 g		
Solution 3				
Ferric tartrate bubble in & sit overnight	$FeC_4H_4O_6.3H_2O$	0.25 g	100-ml H_2O	1-ml
Other solutions				
NAA	$C_{10}H_7CH_2COOH$	0.02 g in 95% EtOH	100-ml H_2O	1-ml
2,4-D	$Cl_2C_6H_6OCH_2COOH$	0.25 g	500-ml H_2O	4-ml

Ingredients for one liter:

- Solution 1: 100 ml
- Solution 2: 1 ml
- Solution 3: 1 ml
- Distilled water: 898 ml
- Sucrose: 20 g
- Agar (bacto): 10 g
- NAA solution: 1 ml
- 2,4-D: 4ml

Adjust the pH to 6,5 at 60 °C

Fill the test tubes (20 ml/ tube)

Annex 4: Modified Strullu-Romand medium (MSR).

Composition of the medium:

SOLUTIONS	COMPONENTS		CONCENTATED IONS
Macroelements	$MgSO_4.7H_2O$		7.39 g/l
	KNO_3		0.76 g/l
	KCL		0.65 g/l
	KH_2PO_4		0.041 g/l
Microelements	$MnSO_4.4H_2O$	*	1.225 g/500ml
	$ZnSO_4.7H_2O$		0.14 g/500ml
	H_3BO_3		0.925 g/500ml
	$CuSO_4.5H_2O$		0.11 g/500ml
	$Na_2MoO_4.2H_2O$		0.0012 g/500ml
	$(NH_4)_6Mo_7O_{24}.5H_2O$		0.017 g/500ml
Vitamins	Panthotenate Ca		0.09 g/500ml
	Biotine		0.1 g/500ml
	Nicotine acid		0.1 g/500ml
	Pyridoxine		0.09 g/500ml
	Thiamine		0.1 g/500ml
	Cyanocobalamine		0.04 g/500ml
Calcium nitrate	$Ca(NO_3)_2.4H_2O$		1.795 g/500ml
NaFeEDTA	NaFeEDTA		0.16 g/100ml

* Preparation of the micro-elements solution:

- Dissolve 1,225g $MnSO_4.4H_2O$ in 100 ml distilled water
- Dissolve 0,14g $ZnSO_4.7H_2O$ in 100 ml distilled water
- Dissolve 0,925g H_3BO_3 in 100 ml distilled water
- Mix those three solutions
- Dissolve 1,1g $CuSO_4.5H_2O$ in 50 ml distilled water and pipette 5 ml in the solution
- Dissolve 0,12g $Na_2MoO_4.2H_2O$ in 100 ml distilled water and pipette 1 ml in the solution
- Dissolve 1,7g $(NH_4)_6Mo_7O_{24}.5H_2O$ in 100 ml distilled water and pipette 1 ml in the solution
- Add distilled water up to a final volume of 500 ml

Preparation of 1 liter MSR medium:

- Put 700 ml distilled water in a one-liter recipient
- Add 100 ml of the macroelement solution
- Add 100 ml of the calcium nitrate solution
- Add 5 ml of the NaFeEDTA
- Add 1 ml of the microelement solution
- Add 5 ml of the vitamin solution
- Add 10g of saccharose
- Add distilled water up to a volume of 1 l, and stir well
- Adjust the pH to 5.5
- Add 8 g of bacto-agar
- Autoclave the medium

Annex 5 : 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 6: Relationship between the geometric mean of nematode number per plant at 6 and 8 weeks and fresh root weight at 12 weeks after inoculation of banana plants (*Musa* AAA cv. Poyo) with 100 *Radopholus similis* (Fallas & Sarah, 1995). Abbreviations on the figure represent nematode populations from Sri Lanka (SRL), Queensland (QUE), Guadeloupe (GUA), Cameroon (CAM), Nigeria (NIG), Guinea (GUI), Ivory Coast (CIV) and Uganda (UGA). CTR indicates untreated control.