Université catholique de Louvain

Faculté d'ingénierie biologique, agronomique et environnementale

Earth and Life Institute

Applied Microbiology (ELIM)

Laboratory of Mycology

Impact of culture conditions on root growth and mycorrhization of *Hevea brasiliensis in vitro*

Thèse de doctorat présentée en vue de l'obtention du grade de Docteur en Sciences agronomiques et ingénierie biologique

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Figure 1. Récolte du latex par saignée de l'hévéa (Cambodge). Ph. © Thierry Falise / Gamma [http://www.larousse.fr/encyclopedie/nom-commun-nom/caoutchouc/30471]

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List of abbreviations

AC	Activated charcoal
ACP	Acid phosphatase
ALP	Alkaline phosphatase
AM	Arbuscular mycorrhizal (fungi)
AMF	Arbuscular mycorrhizal fungi
AM-P	Arbuscular mycorrhizal-plant system
ANOVA	Analysis of variance
ECM	Ectomycorrhizal (fungi)
ERM	Extraradical mycelium
ESR	Early secondary roots
HAM-P	Half-closed arbuscular mycorrhizal-plant system
HC	Hyphal compartment
IBA	Indole-3-butyric acid
MDP	Mycelium donor plant
MES	2-(N-Morpholino)ethanesulfonic acid sodium salt
MSR	Modified Strullu-Romand medium
NBT	Nitro blue tetrazolium chloride syn. Nitrotetrazolium blue chloride
NR	Natural Rubber
RC	Root compartment
ROC	Root organ culture
SDH	Succinate dehydrogenase
SRAS	Secondary roots of the acropetal sequence
UDA	*Urtica dioica* agglutinin

Glossary

Acclimatization: Transplantation of *in vitro* produced plants to *ex vitro* culture conditions (e.g. greenhouse, nursery and field). Plantlets that have grown *in vitro developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow heterotrophic growth, and in an atmosphere with high level of humidity. These contribute to a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field* (Hazarika 2003).

Anti-Microbial Peptides (AMPs)*:* Diverse collection of molecules grouped in different structural families, which are part of the innate immunity of the plants, establishing a first line of defence against pathogens. All plant organs express AMPs constitutively or in response to microbial challenges. The main classes of AMP are: thionins, defensins, lipid transfer proteins (LTPs), snakins, knottins, cylotides and **hevein-like AMPs** (Odintsova and Egorov 2012; Stotz et al. 2013).

Arbuscular mycorrhizas (AM): *Unique interaction between two eukaryotes – an obligate biotrophic fungus and its host plant – leading to an overall improvement of the fitness of the interacting partners. AM improve plant nutrient uptake thanks to the fine exploration of the rhizosphere by the hyphae, which in return receive plant carbohydrates that are essential for the completion of the fungal life cycle* (Bonfante and Genre 2008).

Arbuscule: *Intraradical structure characteristic of the arbuscular mycorrhizal symbiosis consisting of a fungal cell that penetrates and ramifies inside the cortex of a plant cell. Each fungal branch is surrounded by a plant-derived periarbuscular membrane (PAM) that excludes the fungus from the plant cytoplasm. The nutrient exchange takes place in the interface formed between the fungal cell membrane and the PAM, called the periarbuscular space – PAS* (Parniske 2008).

Biotization: *Metabolic response of* in vitro*-grown plant material to (a) microbial inoculant(s), leading to the developmental and physiological changes enhancing biotic and abiotic stress resistance of the derived plant propagules. Establishment of artificial symbiotic associations could also belong to this category* (Nowak 1998).

Extraradical mycelium: *Fungal hyphae network that explores the substratum (soil, culture medium…) to efficiently take up nutrients (phosphates, ammonium, zinc..) and water* (Parniske 2008).

Hevein: *Is a small (4.7 KDa) cysteine-rich protein present in the latex of the rubber tree. It is one of the major proteins in the lutoids, which are small vacuole-derived organelles.* Is a chitin-binding protein which strongly resembles the lectin from stinging nettle (*Urtica dioica* L). *Like the latter, hevein showed strong antifungal activity against several fungi in vitro* (Van Parijs et al. 1991).

Hyphopodium: *Modified (usually distended and branched) AM hypha that is attached to the host plant epidermis to penetrate the root plant cells and to start the intraradical colonization. This term is currently replacing the term apressorium* (Bonfante and Genre 2008).

Intraradical mycelium: *AM fungal component composed by the hyphae that extend within the intercellular root space of the root epidermis and cortex, branching and anastomosing, forming specific structures as arbuscules, coils and in some species, also vesicles and spores* (Dalpé et al. 2005).

Latex: Is the product extracted by tapping the bark of the natural rubber tree, processed to obtain raw natural rubber. *From a cell biology point of view, latex is essentially the cytoplasmic content of laticifers or latex vessels. As cytoplasm, latex contains organelles typical of eukaryotic cells but of particular interest to rubber biosynthesis are the latex-specific organelles i.e. rubber particles and Frey-Wyssling particles. The Frey-Wyssling particle is a specialized plastid which contains carotenoids thus giving a yellowish color to the latex of some rubber tree clones* (Chow et al. 2012).

Micropropagation: In vitro *vegetative propagation of plants.* Depending on the technique applied, *the products of such micropropagation are usually called plantlets, somatic embryos, or micropropagules* (Ahuja 1993).

Mycotrophy* or *Mycorrhizal dependency: *Plant species differ in their ability to grow without AMF at different level of mineral availability (*Janos 1980*).* **Non mycorrhizal -NM plants** (also called **non-host plants**), can grow normally without forming mycorrhizae, even at low P levels. **Mycorrhizal** (or **mycotrophic**) plants associate to mycorrhiza and their growth is stimulated by the symbiosis, but their dependency to the association differs; some of them have the ability to continue growth without forming mycorrhiza, although colonization improves growth (**facultatively mycorrhizal**), and other species die if they are not colonized (**obligately mycorrhizal**) (Brundrett 2002; Janos 1980; 2007).

Facultatively mycotrophic species (or **facultatively mycorrhizal**) *can attain reproductive maturity without mycorrhizae in the more fertile of their natural habitats. They form fewer mycorrhizae in fertile soils than in poor soils* (Janos 1980; 2007).

Obligately mycotrophic species *(or* **obligately mycorrhizal***)* are defined *as those that cannot survive to reproductive maturity if uninfected at the fertility levels encountered in their natural habitats. Although obligately mycotrophic plants may be able to grow without mycorrhizae if heavily fertilized, some tropical trees may be incapable of growth without mycorrhizae under any mineral regime* (Janos 1980; 2007).

Natural rubber: *Natural rubber is found in the latex of the* Hevea brasiliensis *tree and consists of high molecular weight cis-polyisoprene produced from the isoprenoid pathway* (Chow et al. 2012). *Is the raw material for the manufacture of a very large number of finished products* (e.g. *strong structural materials, resilient elastic materials…, nonconductors of electricity, shock absorbers…, clothing articles…, pharmaceuticals… and the most important, the manufacture of tires…;* Nair 2010).

Phosphatase enzymes: *Enzymes that hydrolyze P esters* into inorganic phosphorus (Joner et al. 2000). In the arbuscular mycorrhizal fungi, *they are implicated in the fungal P metabolism (i.e. fungal nutrition) and the intracellular P transport and transformation (*van Aarle and Olsson 2008). Alkaline phosphatase (EC 3.1.3.1) -ALP activity in the extraradical mycelium has been associated with the P uptake from the rhizosphere. Acid phosphatase (EC 3.1.3.2) -ACP activity in the intraradical mycelium has been associated with the transfer of P from the mycelium to the host plant.

(Photo) autotrophic micropropagation: *Plant tissue culture technique for the massive propagation of plants made without sugar in the culture medium, in which the growth of the cultured plants only depends of photosynthesis and inorganic nutrients uptake* (Xiao et al. 2011).

Somatic embryogenesis: *Micropropagation technique consisting of the* in vitro *regeneration of a plant embryo from different plant adult vegetative tissues (e.g. anther wall, inner tegument of the seed), involving several successive phases* of in vitro *culture* (e.g. *callogenesis, differentiation, multiplication, development of the embryos into plantlets*; Carron et al. 2009).

Succinate dehydrogenase: *Enzymatic activity used as index of fungal metabolic activity. SDH enzyme acts in the tricarboxylic acid (TCA) cycle, consequently, its measurement can be used as an indicator of the respiratory fungal activity* (Saito 1995). SDH (EC 1.3.99.1) is an enzyme complex bound to the inner mitochondrial membrane. In the TCA cycle, SDH catalyzes the oxidation of succinate to fumarate (SDH + succinate <=> SDH H_2 + fumarate). In the reaction for the vital stain, NBT acts as the substrate for the SDH enzyme. The salt is reduced into insoluble NBT formazan.

Urtica dioica agglutinin: *UDA is a single-chain peptide found in roots and rhizomes of stinging nettle (*Urtica dioica *L.). In most ecotypes, UDA is present as a mixture of isolectins with similar chitin-binding and agglutination activities* (Van Damme et al. 1988, Does et al. 1999). *UDA consists of two Cys-rich chitin-binding domains. These domains are homologous to hevein, a small chitin-binding peptide of 43 amino acids from the lutoid bodies of rubber tree latex* (Does et al. 1999).

Summary

Hevea brasiliensis is a tree cultivated worldwide for the commercial production of natural rubber. Nowadays, genetic plant improvement coupled to micropropagation techniques is applied to optimize the production of clones with high-yield capacity. Micropropagated plants also represent an important source of selected material that may be used for specific studies (e.g. *in vitro*) of the interaction between this plant and beneficial soil microorganisms as the arbuscular mycorrhizal fungi (AMF).

AMF play an important role in the improvement of the plant growth and the maintaining of the soil quality, and can increase the resistance of *in vitro* produced species against abiotic and biotic constraints that could stress them during the acclimatization phase. Furthermore, the inoculation of AMF on rubber seedlings in greenhouse and nursery has been shown to increase the biomass and the nutrient accumulation of young plants. However, the positive effects of the inoculation on the plant growth are not immediate, and the colonization of the roots only starts before several weeks.

Therefore, *in vitro* studies on the mycorrhization of *H. brasiliensis* are essential to increase the knowledge about the biology of the symbiosis during the first weeks of contact between the partners, and to validate the utilization of AMF during the early stages of growth of the rubber plants in the nursery. The objective of this thesis was to study the effect of the culture conditions on the association between the AMF *Rhizophagus irregularis* MUCL 41833 and *H. brasiliensis* clone PB 260 plantlets growing in an autotrophic *in vitro* culture system.

In a first step, a half-closed arbuscular mycorrhizal-plant autotrophic *in vitro* culture system was adapted to produce colonized plantlets in a sugar and vitamin-free medium. The impact of high atmospheric CO_2 concentration in the growth chamber, pre-treatment of the plantlets with indole-3-butyric acid -IBA, and buffering with 2-(N-morpholino)ethanesulfonic acid sodium salt (MES) were evaluated. The combination of high atmospheric CO_2 (1000 μmol mol^{-1}) and addition of MES (10 mM) to the culture medium improved the *H. brasiliensis* root growth and colonization by the AMF. However, the time needed for adequate root colonization was too long (approx. 7 weeks) to be integrated into a standard *in vitro* culture cycle.

In a second step, the application of activated charcoal (AC) to the growth medium, and the pruning of the taproot of the *H. brasiliensis* plantlets were assayed in an attempt to improve root colonization. The addition of AC enhanced the growth of the roots and AMF colonization, without having negative consequences on the fungal metabolism (SDH and ALP enzymatic activities). The pruning of the taproot did not significantly impact root elongation or AMF viability, but had a negative effect on total root colonization. The treatments did not accelerate the formation of new roots or the root colonization, suggesting that other factors may be involved in the control of root emission and/or the early and fast mycorrhization.

In a third step, we hypothesized that the delay in root colonization may be related to root exudates produced by *H. brasiliensis*, which have antifungal properties. To test this hypothesis, we placed *Medicago truncatula* seedlings in an actively growing extraradical mycelium network of *R. irregularis*, in close vicinity of a *H. brasiliensis* plantlet. We used *Urtica dioica* as control, since this plant in able to disrupt the colonization of neighboring plants by exudation of the antifungal protein *Urtica dioica* agglutinin -UDA. This protein has a close similarity with hevein, a protein present in the latex of *H. brasiliensis*. We observed a decrease in the root colonization of *M. truncatula* in presence of both plants, and a negative effect on the metabolic activity of the AMF, suggesting that roots exudates containing antifungal molecules were produced.

In conclusion, we successfully associated *H. brasiliensis* plantlets to the AMF *R. irregularis in vitro*. We established the culture conditions that were the most appropriate to obtain improved *in vitro* root growth and colonization. However, we also observed that the production of new roots and their subsequent mycorrhization appear to be highly controlled by the plant. The experimental system adapted during this thesis offers large opportunities for molecular and physiological studies on this symbiotic association that could contribute to elucidate the factors (e.g. plant genetics, plant secondary metabolism…) that are regulating the first steps of the symbiosis.

Outline of the thesis

The aim of the thesis was to establish the influence of the culture conditions on the root growth and the colonization with *Rhizophagus irregularis* MUCL 41833 of *Hevea brasiliensis* clone PB 260 *in vitro*. The thesis was developed in four chapters which are summarized below.

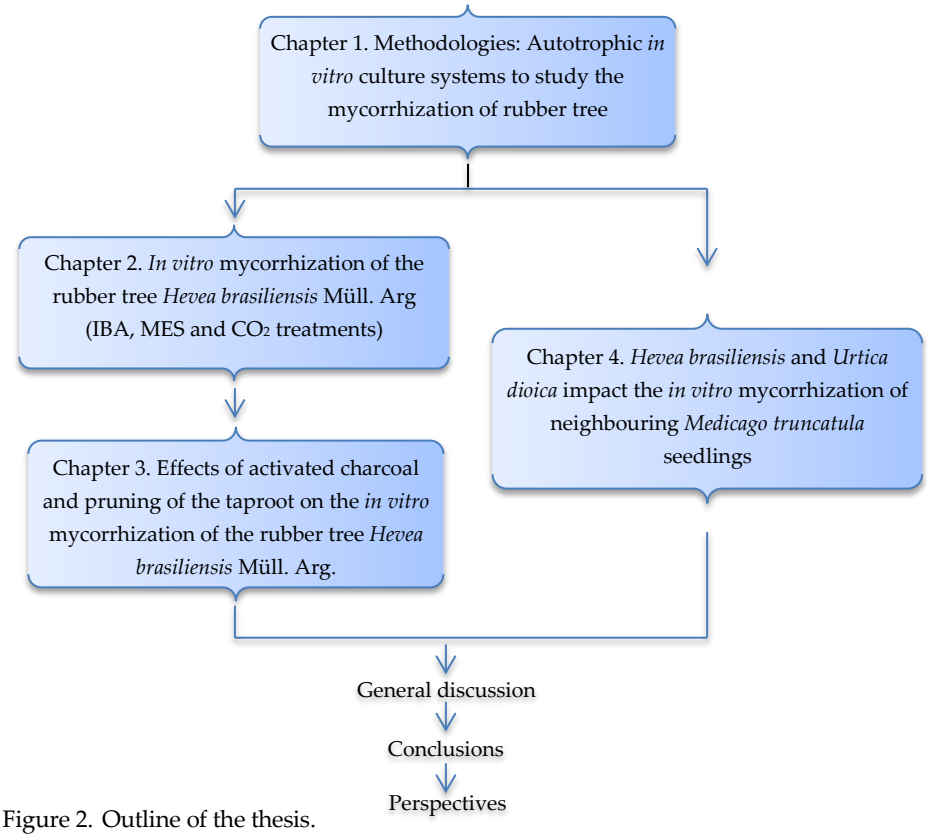

Figure 2. Outline of the thesis.

The **introduction** section presents the subject and the general objective of the thesis. In the **State of the art** section, we reviewed the main aspects approached in this research. We summarized the principal characteristics of *H. brasiliensis* and its culture. We also focused on the symbiosis with AMF and the *in vitro* culture of these obligate symbionts.

The half-closed arbuscular mycorrhizal plant (HAM-P) *in vitro* culture system was originally developed to allow the autotrophic culture and mycorrhization of *Solanum tuberosum* (Voets et al. 2005). Similarly, the

arbuscular mycorrhizal plant (AM-P) *in vitro* culture system allowed the mycorrhization of *Medicago truncatula* (Dupré de Boulois et al. 2006). Both plants have limited secondary growth. *H. brasiliensis* is a tree, and for this reason, it was necessary to adapt the *in vitro* culture systems to the secondary growth (Nicole 1984) and to the large size of its shoots and roots.

In **Chapter 1** we summarized the techniques, materials and methods that were specifically used to study the AMF colonization of rubber plantlets. The HAM-P and the AM-P *in vitro* culture systems were successfully adapted for the *in vitro* colonization of rubber tree by direct inoculation of *in vitro* produced AMF spores in the vicinity of the roots. However, with these systems, several weeks were necessary to obtain the mycorrhization. This was partly related to the time needed for the germination of the spores, the growth of the AMF mycelium, and the subsequent contact and colonization of the roots. The mycelium donor plant (MDP) *in vitro* culture system developed by Voets et al. (2009) was therefore assayed to overcome this limitation. This system was shown efficient for the fast (approx. 7 days) root colonization of several plants (Dupré de Boulois et al. 2009; Voets et al. 2009). The MDP *in vitro* culture system is based on the production of an extraradical mycelium (ERM) network of hyphae that remains connected to a host plant (i.e. the mycelium donor plant). Then, a receiver plant (e.g. *H. brasiliensis*) is placed in contact with the ERM to facilitate the rapid root colonization.

Chapter 2 describes the application of the MDP *in vitro* culture system for the colonization of *H. brasiliensis* plantlets by *R. irregularis* MUCL 41833. The plantlets were plated in the actively growing mycelium. Several factors (Indole-3-butyric acid -IBA, 2-(N-Morpholino)ethanesulfonic acid sodium salt -MES, and carbon dioxide -CO_2) were tested on *H. brasiliensis* root development and subsequent colonization. Root colonization (%) was obtained within a period of 7 weeks. The highest percentages were obtained under a high (1000 μmol mol^{-1}) CO_2 concentration in a growth medium buffered with 10mM of MES.

The results described in this chapter were published in the journal: "In Vitro Cellular & Developmental Biology - Plant" (Sosa-Rodriguez et al. 2013a)

Notwithstanding the successful *in vitro* association of the AMF with *H. brasiliensis*, the time needed to obtain adequate colonization was considered too long. Therefore, it was important to understand which factors were responsible for this delay. This question was addressed in **chapter 3** and **chapter 4**.

In **Chapter 3,** we investigated which culture conditions may improve root initiation of the rubber plantlets and their subsequent colonization. We evaluated the impact of activated charcoal (AC) and the taproot pruning on the root growth and colonization of *H. brasiliensis*, and the influence of both treatments on the enzymatic activity of the ERM of *R. irregularis*. AC addition and pruning of the taproot were selected as treatments to induce the production of root because their stimulatory effect was already observed (Thaler and Pagès 1997; Thomas 2008). The total root length of the plantlets, as well as the root colonization and the production of arbuscules and intraradical spores/vesicles were significantly increased in presence of AC, while no positive effect was noticed following the taproot pruning. Moreover, none of the treatments induced the apparition of new early secondary roots (ESR). The AMF enzymatic activities (SDH and ALP) were not affected.

The results described in this chapter were submitted to the journal: "In Vitro Cellular & Developmental Biology - Plant"

Since the reason(s) for the slow colonization of *H. brasiliensis* plantlets under *in vitro* culture conditions remained speculative, we hypothesized that root exudates from *H. brasiliensis* may have compounds with antifungal properties, thus interfering with the mycorrhization of the rubber plantlets, and with the root colonization of *M. truncatula*, a highly mycotrophic plant. In **Chapter 4**, *M. truncatula* was grown in the close vicinity of *H. brasiliensis* or *Urtica dioica* (also known to synthetize UDA, an agglutinin with antifungal properties), to study the effect of these plants on the root colonization of *M. truncatula* by *R. irregularis*. The presence of both plants decreased the root colonization of *M. truncatula* and the metabolic activity of the ERM of the AMF, suggesting that diffusible inhibitory molecules were exuded by *H. brasiliensis* and *U. dioica*.

The results described in this chapter were published in the journal: "Symbiosis" (Sosa-Rodriguez et al. 2013b)

In the **General discussion** section, the major findings of the thesis were summarized and interpreted. In the **Conclusions** section, the main outcomes of this research were listed and further commented. Finally, the **Perspectives** section addressed a number of hypotheses and possible strategies to improve the early colonization of *H. brasiliensis in vitro*. We also suggested some avenues to better understand the early steps of root colonization in the association *H. brasiliensis* - AMF.

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Introduction

Natural rubber is the raw material used in the manufacture of about 50000 different industrial products over the world. In addition, secondary products issued from rubber plantations such as wood (main by-product of the rubber plantations), rubber honey or seeds, are commercialized in several countries (Clément-Demange and Dattée 2006). The adoption of vegetative propagation (instead of the propagation by seeds), was one of the main factors that contributed to the rapid extension of rubber plantations and the rapid progress of the rubber industry (Nair 2010). Nowadays, most of the propagation practices are applied in the nurseries. Genetic improvement and mass propagation of selected clones have become major objectives for the industry that require the application of innovative biotechnological tools e.g., the *in vitro* production of improved plantlets and their biotization with microorganism before planting in the fields (Nowak 2008). Indeed, the selection of highly productive clones, and the quality of the starting plant material are essential to increase the latex production (Carron et al. 1995; Nair 2010).

One of the most interesting applications of microorganisms in agriculture is plant bio-stimulation**.** The potential of bacteria and fungi to enhance plant growth and biomass and to improve plant yield have been reported in a variety of commercial crops such as maize, wheat, tomato, potato, banana... The main groups of microorganisms with reported growth stimulation effects are plant growth-promoting rhizobacteria -PGPR (see Compant et al. 2010 for specific examples) and arbuscular mycorrhizal fungi -AMF (Smith and Read 2008).

AMF are widespread soil fungi that form symbiotic associations with the root system of most agricultural crops. They improve plant growth and increase their resistance to biotic (e.g. phyto-pathogenic bacteria, fungi, nematodes…) and abiotic stresses (e.g. drought, salt, aluminum toxicity…; Smith and Read 2008). They are also reported to improve the quality of the nursery plants (e.g. improvement of the plant biomass accumulation) before to the transplantation into the field (Solaiman and Hirata 1997; Porras-Soriano et al. 2009).

For instance, banana plants associated *in vitro* to AMF showed increased biomass during acclimatization the greenhouse (Koffi *personal*

communication). The use of micropropagated *H. brasiliensis* plants in *in vitro* autotrophic culture studies and their association with AMF represent an innovative approach to a better understanding of the biology of this symbiotic interaction. In addition, the early association with AMF could accelerate the transplantation of *H. brasiliensis* nursery plants to the field.

Moreover, the utilization of microorganisms to stimulate the production of plant by-products other than food is also possible. For instance, AMF has been applied to stimulate the production of plant secondary metabolites, essential oils and medical compounds (Khaosaad et al. 2006; Gianinazzi et al. 2010). During this thesis we studied the culture conditions that could improve the association of *H. brasiliensis* plantlets with AMF *in vitro*. In the near future, this model may contribute to the understanding of a possible role of these microorganisms on the latex production of the rubber trees.

In the recent years, the *in vitro* cultivation of different autotrophic mycorrhizal plants (potato, barrel medic, banana…) has been developed in the Laboratory of Mycology of the Université catholique de Louvain -UCL (Voets et al. 2005 and 2009; Dupré de Boulois et al. 2006; Gallou et al. 2010; Koffi et al. 2012). This methodology offers the advantage to be contaminant-free and is ideal for fundamental research on the physiology of the plant-AMF association. It further opens the door to mass production of *in vitro* mycorrhizal plantlets, thus potentially improving their resistance at transplantation to the hardening phase. The practical application of this technology for enhancing the quality of *H. brasiliensis* plantlets during the micropropagation phase of selected clones has never been assayed so far.

The objective of our thesis was to study the *in vitro* association between *H. brasiliensis* clone PB 260 and the AMF *Rhizophagus irregularis* MUCL 41833. The mycelium donor plant *in vitro* culture system developed by Voets et al. (2009) was adapted to the rubber plantlets. Using this system, we investigated the dynamics of root production and colonization by the AMF. The enzymatic activity in the extraradical mycelium of the AMF was also monitored. In addition, we tested the effect of several culture conditions (e.g. atmospheric CO_2 concentration, addition of MES buffer or activated charcoal to the culture medium, IBA plantlets pre-treatment, taproot pruning…) on root growth and colonization. Finally, we hypothesized that root exudates of *H. brasiliensis* plantlets may impact their colonization as well as the colonization of neighboring highly-mycotrophic plants (i.e. *M. truncatula*) at the early stages of the symbiosis.

State of the art

Latex and natural rubber

Natural latex is extracted from rubber trees. It is composed by rubber particles (35-40% of the volume), water, carbohydrates (e.g. quebrachitol, sucrose, and glucose), proteins (1%), lipids (1.6%), inorganic salts (e.g. K, Mg, Cu, Fe, Na, Ca, P) and other minor substances (Nair 2010).

From the cellular point of view, latex is synthetized and contained in the laticiferous system, a tissue originated by the activity of the vascular cambium, present in the bark of the rubber tree (Nair 2010). Latex is the cytoplasmic content of the latex cells. As cytoplasm, it contains organelles typical of eukaryotic cells, but also contains latex-specific organelles as the rubber particles, the Frey-Wyssling particles (specialized plastid which contains carotenoids), and a specialized type of lysosomes, the lutoids, subcellular membrane-bound bodies that enclose a fluid serum which contain organic and mineral solutes and several distinct proteins (Archer 1960; Chow et al. 2012; Nair 2010). Some of them have chitinase/lysozyme enzymatic properties (Martin 1991). Others, such as the hevein-like antimicrobial peptides, can bind to chitin and have shown antifungal activity (Odintsova and Egorov 2012; Van Parijs 1991).

Natural rubber (NR) is a high molecular weight cis-polyisoprene produced from the isoprenoid pathway (Chow et al. 2012). It belongs to an assembly called "the large tonnage rubbers" grouping those rubbers of minimal costs that can be used to make tires and other "general rubber goods". NR is a reference standard for the entire rubber industry because of its performance properties (high strength and excellent resistance to dynamic flexing). Its technical weaknesses (e.g. undesirable resistance to heat, light and atmospheric ozone…) are also shared with several synthetic rubbers (Allen 1972; Nair 2010).

According to the International Rubber Study Group -IRSG, 92% of the world natural rubber is produced in Asia (Thailand, Indonesia, Malaysia, India, Chine, Cambodia and Vietnam), 4,5 % in Africa (Liberia, Ivory Coast, Nigeria, Cameroon, and Gabon) and 3,5 % in Latin America (Mainly in Brazil and Guatemala; IRSG 2012). The rubber tree is also cultivated in Colombia, where it covers approximately 28000 hectares (Silva 2011).

The rubber tree

Hevea brasiliensis Müll. Arg. (*Euphorbiaceae*) is a tree native from the Amazon basin in South America (Bolivia, Brazil, Colombia, Ecuador, Peru and Venezuela), used for the production of commercial natural rubber due to its high latex production capacity (Delabarre and Serier 1995).

Figure 3. Tapping process on *Hevea brasiliensis* tree to produce latex. [http://medicalstar.net/latex%20gloves-eng.html]

In natural forest, rubber trees can reach 40 m height but in plantations their size is smaller (approx. 20-30 m), because of the latex tapping. The economic life time of a rubber tree in plantation is around 32 years, with 7 years of immature non-productive phase, and 25 years of latex productive phase (Compagnon and D'Auzac 1986; Carron et al. 1989; Clément-Demange and Dattée 2006).

Figure 4. *Hevea brasiliensis* fields (Remolino - Meta, Colombia).

The shoot part of the rubber tree has a rhythmic growth. The young plants show characteristic growth patterns of alternating periods of rapid elongation and consolidated development (Nair 2010). During its vegetative growth, the natural rubber tree loses and renews its foliage each year. The duration of this phenomenon is about sixteen weeks.

H. brasiliensis is a monoecious plant with unisexual flowers produced in pyramid-chapped panicles in the axils of leaves having numerous small male flowers and fewer female flowers of bigger size. The female flowers are confined to the tip of the panicles and their branchlets. The ovary is tricarpellary syncarpous, which develops into a three-lobed dehiscent capsule with three large, mottled seeds (Nair 2010).

Flowers are produced during the re-foliation step. In plantations, the first flowers appear in 4 – 5 year-old trees, producing seeds that have a short viability (Delabarre and Serier 1995). Flowering occurs during the dry season. In tropical regions with a monomodal rainfall regime (one wet and one dry season) rubber trees produce seeds once a year, whereas in tropical regions with a bimodal rainfall regime (two wet and dry seasons), seeds are produced twice a year (Martínez et al. 2006).

The tree reproduction is accomplished by cross-pollination, usually done by insects (e.g. bees, midges and thrips; Delabarre and Serier 1995; Yeang and Chevallier 2009; Nair 2010), wind (Montoro, *personal communication*) or manually (i.e. hand pollination; Sedgley and Attanayake 1988). The seeds are ovoid and 2 to 3.5 cm diameter. One kg of seeds can contain 180 to 350 units. The seed loses its germinative power rapidly because of its high oil content that oxidize quickly. Decrease in water content is also detrimental to germination. For these reasons, the seeds should be used within 3 – 4 weeks following harvesting (Delabarre and Serier 1995; Nieto and Rodríguez 2003, Nair 2010).

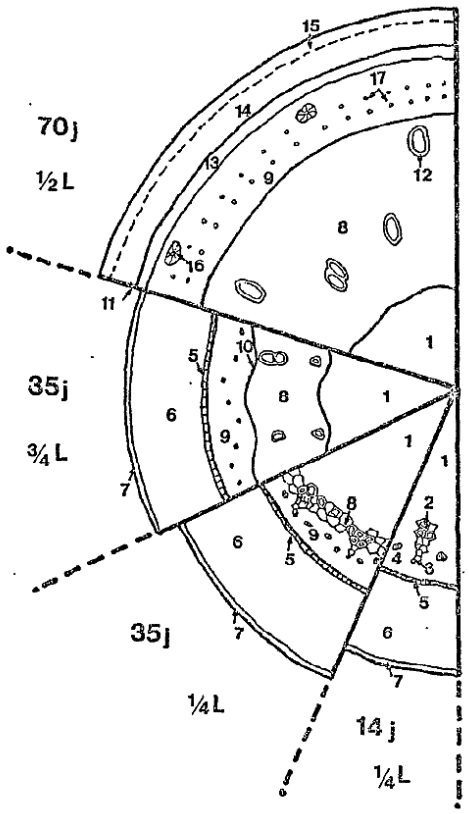
The root system

During the early development of a young rubber seedling, i.e. the first 40 days after the seed germination, different types of roots emerge. According to the spatial and temporal location of emergence, five different types can be distinguished: (1) taproot; (2) early secondary roots -ESR; (3) secondary roots of the acropetal sequence -SRAS; (4) late secondary roots -LSR; (5) tertiary roots (Le Roux and Pagès 1994; 1996).

The taproot is the primary axis of the root system; it has a strong dominancy, with vertical geotropic growth. This root has a continuous growth that can

vary from 22 mm/day (first 25 days) to 12 mm/day (next days). The early secondary roots -ESR, appear in the basis of the taproot (junction between the stem and the taproot) 5 to 6 days after the germination of the seed, forming a whorl of twelve ESR (on average). They grow obliquely or perpendicularly to the taproot. Most of these roots only grow during the first 10 to 20 days after germination, and progressively die. Nevertheless, in some seedlings has been observed that sometimes, certain ESR can grow permanently. The secondary roots of the acropetal sequence -SRAS, appear continuously during the elongation of the taproot perpendicularly to this root, starting 15 to 18 days after seed germination. The late secondary roots -LSR appear in the basis of the taproot 27 to 40 days after germination, in a variable number (1 to 4 per plant), and probably other appears later. The tertiary roots emerge from the SRAS and have a limited elongation (about 3 to 10 days after their apparition), reaching 50 mm (on average), without ramifications (Le Roux and Pagès 1994).

The anatomy of the secondary and tertiary roots is constant. The secondary roots present an apical diameter that varies from 0.6 to 0.8 mm. The tertiary roots present an apical diameter of 0.4 mm (on average). These roots do not present secondary growth (Le Roux and Pagès 1994; Putranto et al. 2012). In contrast, the taproot shows important variations during the first weeks of development. The evolution of the anatomy of a taproot of *H. brasiliensis* during 10 week after the germination of the seed is shown in Figure 5. After 70 days, the secondary structures are completely differentiated (Nicole 1984).



1. Medullary parenchyma
2. Metaxylem
3. Protoxylem
4. Primary phloem
5. Endoderm
6. Primary cortical parenchyma
7. Periderm
8. Secondary xylem
9. Secondary phloem
10. Cork cambium
11. Phellogen
12. Xylem vassels
13. Phelloderm
14. Young cork
15. Old cork
16. Sclerotic cells
17. Laticifers

Figure 5. Evolution of the anatomy of a young taproot of *Hevea brasiliensis* (Adapted from Nicole 1984).
j: days; **L**: length of the taproot measured form the apex

The same root morphology and anatomy is found in older *H. brasiliensis* plantlets. Seven-month-old rubber plants have a strong taproot and several lateral roots. The taproot keeps their dominancy, presenting a large apical diameter, a well-developed stele and primary and secondary growth. Lateral roots do not present secondary growth. Interestingly, each type of roots has a specific patron of genes expression that suggests the specialization of the root system to accomplish different functions (Putranto et al. 2012). Indeed, the root system of an adult rubber tree keeps the root hierarchy, with the taproot which gives stability and anchorage to the plant, playing a role in the longitudinal conduction of water and nutrients, and a superficial network of lateral roots, mostly involved in nutrient uptake, with a short

lifespan and capable of rapid regeneration, thus being involved in fast responses to environmental signals (Delabarre and Serier 1995; Carron et al. 2000; Putranto et al. 2012).

Figure 6. *Hevea brasiliensis* (Willd. Ex A. Juss) Mull. Arg. botanical example. Image processed by Thomas Schoepke [http://pharm1.pharmazie.uni-greifswald.de/allgemei/koehler/koeh-071.jpg]

The propagation

At the beginning of the natural rubber industry in South East Asia, seeds were used to establish the plantations, but the seedlings produced had large variation in both growth and latex yield. Grafting techniques were introduced during first years of the 20th Century to achieve vegetative propagation of the most productive trees, contributing to the rapid growth of the rubber plantations with genetically improved clones (i.e. with high latex production and resistance to pathogens; Delabarre and Serier 1995; Nair 2010).

During the grafting process, unselected seeds (i.e. without known parental origin) are germinated and raised in the nursery. The seedlings produced are called rootstocks or patterns. Then, a bud (also called scion) is grafted on the rootstock. The buds come from selected clones that grow in specific areas of the plantation called *clonal gardens*, where only *mother trees* highly

productive and resistant to diseases are propagated (Nieto and Rodríguez 2003; Cardinal et al. 2007). The grafted rubber plants (also called stumps) can be finally transplanted in the field after several months in the nursery (Udayakumara and Seneviratne 2005).

Depending on the age of the buds ant the rootstocks, there are two types of budding: brown and green budding. In brown budding, which is the most extended technique, seedlings of about one year used as rootstock, are grafted with buds collected from mature shoots of the same age. In the green budding, 3 to 5-month-old rootstocks are grafted with young buds (aged of 6 to 12 weeks) (Delabarre and Serier 1995; Nair 2010).

Figure 7. *H. brasiliensis* grafted rootstock (with clone RIMM 600 buds). See the detail of the grafted bud on the right.

In vitro culture of *H. brasiliensis*

Nursery grafting remains the most common technique for the vegetative propagation of natural rubber. A high genetic homogeneity of the grafted plants is expected, because they are generated by vegetative reproduction from a selected clone. Nevertheless, the trees produced by this technique can express undesirable heterogeneity in growth and/or in latex yield, and lower latex production than the mother tree, mainly due to the influence of the rootstock (Carron et al. 1989). In addition, the unselected nature of the rootstocks implies that there is a large variability on the roots systems of the trees of the commercial plantations (Putranto et al. 2012).

In vitro culture of *H. brasiliensis* was developed in the early 1970's to produce plants from selected highly productive clones avoiding the grafting process,

to produce plants "on their own roots" with reduced genetic variation (Carron et al. 1989). To date, several micropropagation procedures (e.g. microcuttings, primary somatic embryogenesis, indirect secondary somatic embryogenesis, genetic transformation of callus lines, development of rejuvenated budded clones…) have been developed in order to optimize de production of recommended clones, but any of them has current commercial application to the massive multiplication of self-rotted clones (see Montoro et al. 2012 for a comprehensive review).

In vitro culture of microcuttings is a multiplication method based on the *in vitro* rooting of *H. brasiliensis* shoots, originated from tree possible sources: (1) young seedlings; (2) rejuvenated budded clones, or (3) primary somatic embryogenesis (used as rejuvenation method). Nowadays, the combination of primary somatic embryogenesis with microcuttings culture seems to be a strategy with great potential for large scale production (Montoro et al. 2012; Prof. Stefaan Werbrouck, *personal communication*).

Somatic embryogenesis from primary callus (derived from anther tissue or internal tegument tissue from immature seeds) has allowed the production of whole plants of 22 *H. brasiliensis* clones, which exhibited better growth and latex production than conventional budded clones (Montoro et al. 2012). Furthermore, hundreds of plantlets produced using this technique have been planted in the field (Carron et al. 1995; Carron et al. 2009; Granet and Gaurel, *personal communication*).

The whole somatic embryogenesis process to obtain complete rubber plants has five stages of culture: callogenesis, embryogenesis expression, somatic embryo development, embryo maturation and germination of somatic embryos. Each of them has specific medium components (for details, see Carron et al. 1995; Lardet et al. 2007 and Montoro et al. 2012). The culture conditions i.e. hormone supply, calcium concentration or carbohydrate content, have shown to be highly specific to the success of certain phases (Carron et al. 1995; Blanc et al. 2002).

Although somatic embryogenesis has been effectively applied in several *H. brasiliensis* clones (e.g. PR 107, RRIM 600, PB 260…) some of them occasionally showed poor germination and embryo conversion rates. Actually, one of the restrictions for this technique is the quantity of the plants produced by explant and the explant availability over the year (i.e. the seeds), that limit its use for clonal multiplication on a large scale. Currently, the technique is mainly being applied for the production of rejuvenated planting material (Montoro et al. 2012).

Microcuttings and primary somatic embryogenesis are the traditional *in vitro* culture procedures developed for *H. brasiliensis*, but other techniques, as the indirect secondary somatic embryogenesis (for large scale propagation), or the genetic transformation from friable callus lines (for functional gene analysis) has been also established (Montoro et al. 2012). For instance, Leclercq et al. (2012) transformed *in vitro* produced plantlets to induce a better plant tolerance to drought and to oxidative stress. The regenerated plants were able to grow in *ex vitro* conditions expressing the genes introduced. Therefore, the *in vitro* produced *H. brasiliensis* plantlets represent an excellent material for specific studies on the genetics and physiology of the rubber tree.

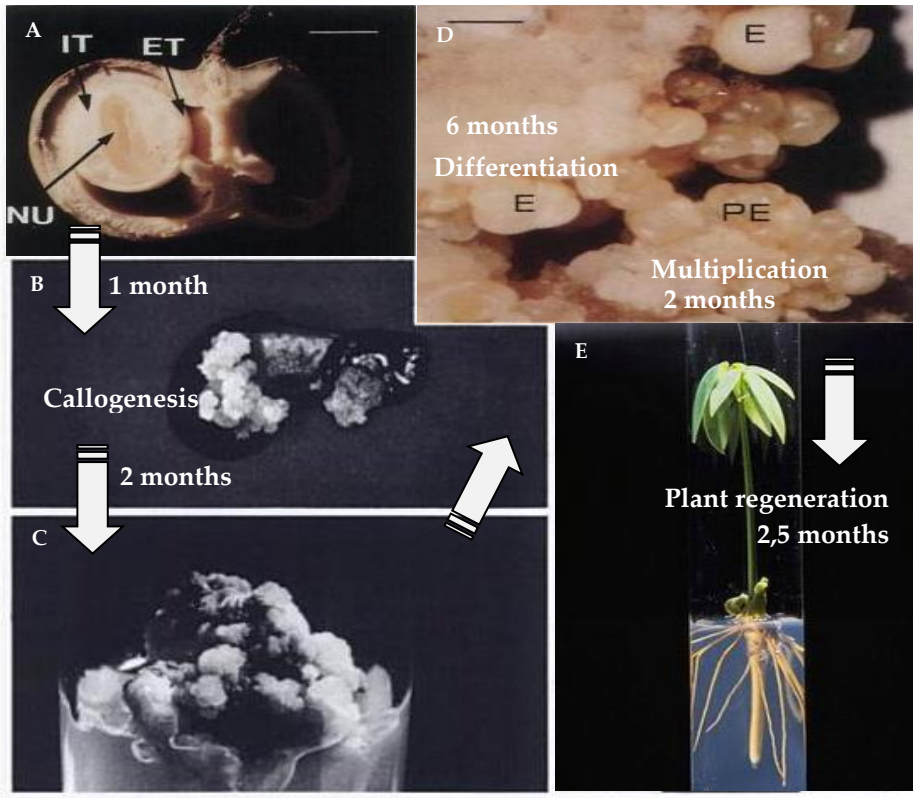


Figure 8. *H. brasiliensis* somatic embryogenesis.
A. Section of an immature fruit with ovule (IT inner integument; ET external integument; NU nucellus) B. and C. Development of callus from the inner integument of the ovule D. Regeneration of multiple embryos (E) on callus and some pro-embryos (PE) E. Development of a plantlet from a somatic embryo (Modified from Carron et al. 1989 and 1995).

The arbuscular mycorrhizal fungi (AMF)

AMF are widespread soil fungi that form symbiotic associations with the root system of 80% of land plants among which most agricultural crops. They improve plant growth, resistance to biotic (e.g. phyto-pathogenic fungi, bacteria and nematodes) and abiotic stresses (e.g. drought, aluminum toxicity), as well as the resistance of nursery plants to transplantation into the field (Liu and Yang 2008; Smith and Read 2008). These observations have been reported for a number of tropical crops such as agave, banana, cocoa, coffee, pepper, pineapple…

The symbiosis is based on the exchange of carbohydrates and minerals between the plant and fungal partners (Figure 9): The AMF forms a network of hyphae in the soil, which takes up minerals (e.g. phosphorus, nitrogen and sulphur) and water from the soil matrix, and translocates these elements to the roots. To start the colonization, specific penetration structures (hyphopodia) are formed **[1]**. In the root cortex, minerals are transferred to the plant cells via arbuscules **[2]**. In parallel, carbohydrates produced by the plant photosynthesis are transferred via these arbuscules to the fungal cells and further redistributed and stored in the mycelium developing within the roots (e.g. intraradical spores/vesicles), **[3]** and outside the roots in the surrounding soil environment for the colonization of new plant hosts.

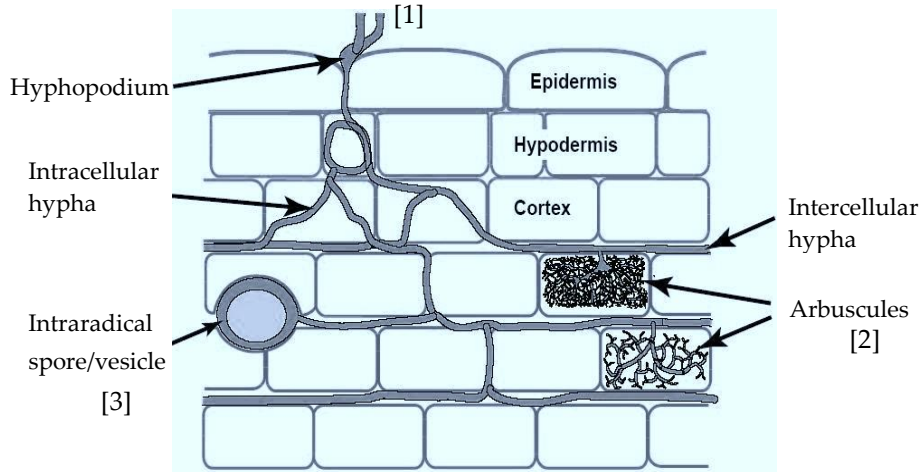


Figure 9. Intraradical colonization of root tissues by an AMF.
[1] Extraradical mycelium [2] Arbuscules [3] Intraradical mycelium and storage structures (Intraradical spores/vesicles). Adapted for Brundrett et al. (1996)

Early interaction between plants and AMF[1]

AMF spores germinate in soil producing a presymbiotic mycelium that explores the soil searching for a host root. The perception of the host plant is mediated by root exudates, which contain attractive compounds identified as strigolactones (Akiyama et al. 2005). These substances only diffuse a short distance, enough for signaling root proximity and stimulate hyphal metabolism and branching.

AMF presymbiotic mycelium is also reactive during the recognition process. It produces specific diffusible molecules that are perceived by the plant even in the absence of physical contact with the fungus. These diffusible compounds are usually referred as ''Myc factors". It is known that they are smaller than 3kDa, partially lipophilic, and probably possessing a chitin backbone. The whole recognition process has not been fully described, but it was recently confirmed that some Myc factors are capable to induce plant membrane-steroid binding proteins, which have shown to be critical for mycorrhization (see Bonfante and Genre (2010) for more details).

Plant responses to Myc factors range from the molecular to the organ level, and are under the control a signal-transduction pathway partly shared with the *Rhizobium*-legume symbiosis, the SYM pathway. It prepares the plant for successful association, controlling pre-contact responses and first steps of colonization of the epidermal and subepidermal cell layers of the plant root. The central factor of the pathway is the calcium ion: the proximity of AMF branched hyphae can induce Ca^{2+} oscillations in the Ca flux between the nucleus and the cytoplasm of the host cells. At least seven genes of the pathway are known to be required to the correct develop of the symbiosis. Besides to the common SYM pathway, increasing data support the existence of alternative signaling pathways which can also induce the expression of certain genes involved in plant early response to AMF.

After the initial exchange of signal molecules, the hyphae rich the surface of the plant root. AMF specifically select the location for starting root penetration. They can extend for several centimeters along the root surface forming long hyphae. Then, they swell, flatten on the cell wall of a few epidermal cells and branch repeatedly to develop the hyphopodium. The exact selection mechanism of the penetration point is unknown, but it has been observed that AMF hyphal branching concentrates in the vicinity of

[1] This section is mainly based on the publication of Bonfante and Genre: « **Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis** » (2010, Nature communications 1:48).

young lateral roots, which are also considered as the primary site of AMF colonization.

AMF stimulation of plant defence[2]

AMF have been reported to protect the plants against a wide number of root diseases (see Whipps 2004 for an extended review). However, the mechanisms of bio-protection remain largely unknown. It is currently accepted that improvement of the nutrition, the competitiveness and defence capacities of the host plant are the central mechanisms.

Bio-protection by AMF has been confirmed as a reduction on the incidence and/or the severity of the diseases caused by different soil-borne pathogens such as *Rhizoctonia*, *Fusarium* or *Verticillium* and by Oomycetes such as *Phytophthora*, *Pythium* and *Aphanomyces* (Whipps 2004) and parasitic nematodes such as *Pratylenchus* and *Meloidogyne* (Elsen et al. 2008). The systemic protection at the root level has also been demonstrated against *Phytophthora, Ralstonia* and *Gaeumannomyces* (See Gallou 2011 for a review).

Some of the protective effects conferred to the host plant by the AMF colonization are: induction of hydrolytic enzymes, augmentation of pathogenesis related proteins, and accumulation of salicylic acid and reactive oxygen species. Direct competition between AMF and pathogens at the site of infection, or the competition by plant carbohydrates is also possible. In many cases, the presence of AMF within the root before the contact with the pathogen seems to be important to favor the plant protection. It is probable that the plant regulates the level of mycorrhizal colonization and the level of pathogen infection at the same time. Moreover, different signaling pathways regulating the plant defence response during AMF colonization has been identified. For instance, it has been observed that mycorrhized roots contain higher levels of endogenous jasmonic acid. At the same time, a partial suppression of the salicylic acid plant dependent responses after AMF colonization has been reported (Pozo and Azcon-Aguilar 2007).

The transiently increased expression of plant defence genes during the early stages of root colonization, followed by a decline in their expression has been also reported (See Gallou 2011 for details). Currently data suggest that

[2] This section is mainly based on the review of Gallou, published on his thesis work « **Impact of *Rhizophagus* sp. (syn. *Glomus* sp.) and *Trichoderma harzianum* on the potato resistance against *Rhizoctonia solani* and *Phytophthora infestans*, two major potato pathogens** » (2011, Université catholique de Louvain).

the host plant initially treats symbiotic fungi as potential invaders and activates a defence program, which is then countered by the mycorrhizal symbionts (Zamioudis and Pieterse 2011).

Host plant responses against AMF

Although AMF can colonize the majority of plant species and are present in most terrestrial ecosystems, the plants dependency or mycotrophy (i.e. their ability to grow without AMF at different levels of mineral availability) differs (Janos 1980). Mycotrophic (or mycorrhizal) plants associate to AMF and show growth stimulation. Two groups of mycorrhizal plants can be defined: Obligately mycorrhizal plants, which can die if they are not colonized, and facultatively mycorrhizal plants, which have some ability to continue growth without forming mycorrhiza (especially at high P levels), although colonization improve growth (especially at low P levels) (Janos 1980; 2007; Brundrett 2002).

Some plant species are reported as non mycorrhizal –NM (or non-host), because they can grow at low levels of P content without forming mycorrhizas. Furthermore, the roots of NM plants are unable to develop a true colonization (i.e. with the development of functional arbuscules) both in natural and in controlled conditions (Janos 1980; 2007; Brundrett 2002; Smith and Read 2008).

Non-host plants have developed different strategies to avoid the development of AMF in their roots, and different hypotheses have been proposed to explain this phenomenon: (1) the plant is unable to produce essential signals for triggering the different steps leading to root colonization (Giovannetti and Sbrana 1998); (2) the plant cannot recognize fungal signals sent during the pre-symbiotic stage (Vierheilig and Bago 2005; Parniske 2008) (3) the plant has barriers against the mycorrhizal colonization that are intrinsic to the roots (i.e. related to physiological characteristics of the root cortex or epidermis; Fontenla et al. 1999); (4) the plant produces and exudates inhibitory or antifungal compounds (Vierheilig et al. 1996; Vierheilig and Bago 2005; Stinson et al. 2006) that impair the fungal growth.

Stimulatory or inhibitory signals/compounds produced by the host could act at different stages of the lifecycle of the AM fungi, i.e., during the asymbiotic (from the spore germination, presymbiotic mycelial growth) pre-symbiotic (hyphal branching, hyphopodium formation) or symbiotic phases (root colonization) (Vierheilig et al. 1995; 1996; 2003; Vierheilig and Bago 2005; Stinson et al. 2006; Yun and Choi 2002 Barto et al. 2011).

For instance, the negative influence of several AM non-host plants such as *Stellaria media*, *Chenopodium album*, *Spinaceae oleracea*, *Brassica campestris, B. nigra, Capsella bursa-pastoris, Sisymbrium altissimum*, *Juncus balticus* and *Urtica dioica* on the root mycorrhization of *Pisum sativum* with *Glomus mosseae* has been reported. *G. mosseae* did not colonize the roots of the non-host plants. Moreover, all of them inhibited the mycorrhizal colonization of *P. sativum.* The observed inhibition was explained as a consequence of substances exuded by the non-host plants with a detrimental effect on the mycorrhization process. It was also proposed that the degree of blockage on the mycorrhization could be dependent of the amount of inhibitory substances that reaches and accumulates in the substrate (Fontenla et al. 1999). Vierheilig et al. (1996) also observed that UDA, a monomeric glycoprotein accumulated in the root and rhizomes of the non-host plant *Urtica dioica*, (Peumans et al, 1984) inhibited the growth of hyphae coming from germinated spores of *G. mosseae*. Indeed, UDA agglutinin antifungal activity has been tested against several root pathogens and saprophytic fungi (Van Parijs et al. 1991).

UDA forms part of the hevein-like AMPs (AntiMicrobial Peptides), a diverse collection of molecules grouped in different structural families, which are part of the innate immunity of the plants, establishing a first line of defence against pathogens. All plant organs express AMPs constitutively or in response to microbial challenges. The main classes of AMP are: thionins, defensins, lipid transfer proteins (LTPs), snakins, knottins, cylotides and hevein-like AMPs (see Stotz et al. 2013 or Odintsova and Egorov 2012 for a comprehensive review).

Hevein-like AMPs show structural similarity with hevein, a small protein with antifungal properties that is a major component of the latex of *Hevea brasiliensis* (Gidrol et al. 1994; Van Parijs et al. 1991). Mature hevein consists of 43 amino acids with four disulfide bridges and contains a conserved chitin-binding domain rich in cysteine and glycine residues, which is present in all the hevein-like AMPs. In addition to hevein, other members of this family are lectins, chitinases, some wound-induced proteins, and a number of peptides later classified as "hevein type" AMPs (Odintsova and Egorov 2012; Stotz et al. 2013).

The antifungal activity of the hevein-like AMPs has been reported against both filamentous fungi and yeast. Furthermore, some of these chitin-binding proteins have shown antibacterial and insecticidal activity (see Odintsova and Egorov 2012 for a review). However, the mode of action of this family of compounds is poorly understood. It has been suggested that the capacity to

bind chitin could interfere with the correct assemblage of the fungal wall (Broekaert et al. 1989; Van Parijs et al. 1991). However, this does not explain their activity against chitin-free (e.g. Oomycetes and bacteria) pathogens (Odintsova and Egorov 2012).

To date, there is no published information about the effect of latex, hevein, hevein-like AMP on the AMF growth or in the mycorrhization process, on *Hevea brasiliensis* or other plants, but the activation of some defence mechanisms in rubber seedlings in the presence of AMF cannot be discarded. For instance, Schwob et al. (2000) found that mycorrhization of *H. brasiliensis* seedlings took several weeks before a complete establishment, and reported some transient defence-like reaction caused by the AMF *G. mosseae*.

H. brasiliensis - AMF interaction

Mycorrhization of *H. brasiliensis* in natural forest soils

AMF species diversity, spore abundance in soil and root colonization values of natural rubber trees growing in different types of forests are summarized in Table 1. The values of root colonization of *H. brasiliensis* are usually high in comparison with other plant species growing in the same soils. For instance, Dhar and Mridha (2006) collected the roots of eight different tree species growing in the Madhupur forest (Bangladesh) to estimate their root colonization by the AMF species present in those soils. Rubber tree roots had the highest total and vesicular root colonization.

Similarly, Feldmann (1994) worked in the Amazonian rainforest, and found that root colonization values of *H. brasiliensis* mature trees were always high, even in ecosystems with very heterogeneous abiotic conditions (e.g. fertility and flooding), and AMF presence was more frequent in rubber roots than in other perennial plant of the same forest (*Theobroma grandiflorum*). The presence of AMF in the roots of young *H. brasiliensis* seedlings has been also recorded. Tawaraya et al. (2003) measured the root colonization on several young forest seedlings (one year-old), and found elevated values on the natural rubber seedling roots.

Table 1. Status of the association between *H. brasiliensis* and arbuscular mycorrhizal fungi in natural forest or plantation soils

Site	**Number of Spores**	**% of Colonization**	**Common AMF found**	**Reference**
India (Tripura five years old plantation)	-	68 -88	*Glomus* sp., *Gigaspora* sp.	Deka et al. 1998
Brazil (Mato Grosso - monoculture rubber tree plantation)	Dry season: 784 spores/100 g soil Rainy season: 1569 spores/100 g soil	38 – 84	*Gigaspora* sp. (dominant)	Schwob et al. 1999
Brazil (Terra Firme - Natural rubber tree sites and plantation soils)	Between 2,7 and 22,3 spores/cm^3 soil	52 – 89	*Glomus etunicatum*, ***G. intraradices***, *G. manihotis, G. mosseae, Gigaspora margarita, Sclerocystis rubiromis, Acaulospora* sp., *Scutellospora* sp.	Feldmann 1994

Table 1. (continuation)

Brazil (Natural rubber tree sites in the Amazon rainforest of the North - East part of the Country)	1220 spores/100 g soil	67	Characteristics similar to *Glomus* sp., *G. occultum, G. fascitulatum, G. mosseae, G. ambisporum, G. diaphanum, G. clarum, G. hoi, G. etunicatum,* ***G. intraradices****, G. manihotis* , *Gigaspora decipiens*, *Gigaspora gigantea, Acaulospora* sp., *A. denticultata, A. dilatata, A. morrowae, A. mellea, Entrophospora* sp., *Entrophospora colombiana, Scutellospora* sp,. *S nigra, S. gregaria, S. calospora* and *Sclerocystis rubiformis*		Feldmann et al. 2000
Brazil (Monoculture rubber tree plantations)	360 - 1760 spores/100 g soil	30 -53			
India (16 different locations)		From 428 to 856/100 g soil	67 -92 (in mature plants)	*Glomus* sp., *Gigaspora* sp. and *Acaulospora* sp.	Joseph *et al*. 2002
Indonesia (in peat swamp forest)		-	47 -72 (in seedlings)	-	Tawaraya et al. 2003
Bangladesh (Madhupur forest area)		-	78	*Glomus* sp. (dominant)	Dhar and Mridha, 2006
Colombia (Clonal gardens of a monoculture rubber tree plantation)		1108 - 1185 spores/100 g soil	80,7 – 95 (in 9 years old trees)	*Glomus* sp., *Scutellospora* sp., and *Acaulospora* sp.	Sosa-Rodriguez et al. 2009
Sri Lanka		40 -4000 spores/100 g soil	-	-	Revised by Akond et al. 2008
Malaysia		-	38 - 58 or 0 to 50	-	Revised by Akond et al. 2008

Mycorrhization of *H. brasiliensis* in plantation soils

The presence of AMF has been reported in commercial plantations of *H. brasiliensis*. The species diversity, number of spores and root colonization varied according to the site and agricultural practices (Table 1). Interestingly, the AMF colonization of *H. brasiliensis* in natural ecosystems was different from the one of the trees growing in plantations. The influence of different factors (e.g. the cultural practices, the fertilization regime, the clone type, the presence of pathogens, the abiotic factors…) on the AMF symbiosis with *H. brasiliensis* was studied.

Feldmann et al. (2000) found a significant negative impact of agricultural practices (application of fungicides, herbicides and use of machinery) on the number of spores, the AMF diversity and the percentage of root colonization in *H. brasiliensis*. Similarly, Deka et al. (1998) and Peña (2000) reported a deleterious impact of chemical fertilizers on the root colonization of the rubber trees when applied in excess.

Sosa-Rodriguez et al. (2009) collected roots from different *H. brasiliensis* clones growing in a 9 year-old clonal garden. They observed that the clone type did not influence the intensity of root colonization. The roots showed high percentages of colonization (Table 1), independently of the clone assessed.

Schwob et al. (1999) studied the interaction between AMF and the pathogenic nematode *Meloidogyne exigua* in the soils of a Brazilian rubber tree plantation. The highest levels of AMF root colonization were found in roots collected from rubber trees growing in soils devoid of nematodes. Interestingly, for roots collected from infected soils, the levels of nematode eggs and juveniles were low when the roots were colonized by AMF.

Weather is an abiotic factor that was shown to impact AMF presence in *H. brasiliensis* plantations. Schwob et al. (1999) assessed the yearly climatic variation on the number of spores (Table 1). They found higher quantities of spores during the rainy season (October to March) as compared to the dry season (April to September). In a similar study, Feldmann et al. (2000) found seasonal variations in the AMF number of spores and AMF diversity. Both parameters were significantly lower in the dry season.

Study of the AMF – *H. brasiliensis* interaction under controlled conditions (growth chamber, greenhouse or nursery)

Numerous studies have been conducted under controlled conditions to investigate the effects of AMF on growth, nutrient contents and plant

physiology. Similarly, the effects of phosphorus fertilization on the mycorrhization, and the possible role of the symbiosis in the protection of the plants against plant pathogens were studied.

The mycotrophy of *H. brasiliensis* was confirmed in greenhouse experiments by Feldmann (1994). Rubber seedlings were inoculated with the AMF *Glomus etunicatum*. The roots were intensively colonized, and the growth of the rubber seedlings was stimulated by the fungus, allowing the classification of *H. brasiliensis* as a mycotrophic plant, that showed higher mycorrhizal dependency than *T. grandiflorum* seedlings grown in the same experimental conditions.

Effects on plant growth parameters and nutrient content

The effect of AMF inoculation on the growth of *H. brasiliensis* has been investigated in different pot experiments. Several authors demonstrated that the effects can be AMF-species dependent (Table 2). For instance, Jayartne et al. (1984) compared *Gigaspora margarita*, *Glomus fasiculatus* and *G. mosseae* in a 4.5 months greenhouse experiment. At the end of the experiment, only the plants inoculated with *G. margarita* showed an increase in shoot biomass.

Ikram et al. (1993) tested the inoculation with ten different AMF isolates on rubber seedlings grown on steam sterilized soils with acid pH and low P contents. Only *G. clarum* and *Glomus* sp. produced a significant increase in plant biomass. The authors suggested that differences in the effectiveness of AMF to improve *H. brasiliensis* growth could be related to the life cycle of each fungus. Furthermore, they did not observe a correlation between the root colonization and the plant growth. For instance, some isolates with low root colonization (e.g. *Glomus* sp.), showed the same positive growth response than isolates with high root colonization values.

The growth response of the seedlings to the application of P can depend on the AMF species. Ikram et al. (1993) also observed that *G. intraradices* was the only isolate that positively influenced the growth after P addition. Additionally, an increase in the leaf P content in the plants inoculated with AMF was measured only with some of the strains (e.g. *Entrophospora colombiana*, *Acaulospora spinosa* and *G. margarita*). In contrast, *G. clarum*, which produced the highest root colonization, did not affect the leaf nutrient content.

A better growth response of *H. brasiliensis* to the AMF inoculation when the P level in the substrate was low has been reported several times, using both mixed (Joseph et al. 2002; Ikram et al. 1992) and isolated (Jayartne et al. 1984; Joseph et al. 2002; Diniz 2007) AMF strains.

Greenhouse experiments with a *G. mosseae* isolate showed a particular response of the rubber seedlings (Schwob et al. 1998): the mycorrhizal colonization started 45 day after inoculation and reached a maximum value of 47% after 180 days. Only at that time the plant height became significantly greater as compared to the controls. The root collar diameter was greater in the inoculated plants only in the final stage of the experiment. The authors suggested that the delayed response of the *H. brasiliensis* plants to the inoculation could be attributed to the initial independence of the plants to external nutrient sources (e.g. the seed reserves), or to effects of the initial establishment of the AMF in the roots in the carbon balance of the plants.

Table 2. Arbuscular mycorrhizal fungi inoculation of natural rubber plants under controlled conditions

Experiment Conditions	**% of Colonization**	**Time after inoculation**	**Number of Spores in the inoculum**	**AM fungi inoculum**	**Reference**
Greenhouse, sterilized and not sterilized soil	Sterilized soil: 5 – 53*%; Not sterilized soil: 31 – 63*% *the highest values were only achieved with *Gigaspora margarita*	126 days (4,5 months)	500 per pot	*Gigaspora margarita*, *Glomus mosseae, G. fasciculatum* (foreign)	Jayartne et al. 1984
Field Nursery (not sterilized sandy and clayey soils)	Natives AM fungi: 30.7 – 52; Native AM fungi + mixed inoculum: 43.3 – 50,1	182 days (6,5 months)	not reported	Mix of foreign species (*Glomus* sp., *G. manihotis, G. clarum, G. intraradices., G. macrocarpum, Entrophospora colombiana, Scutellospora calospora*) and natural AM fungi populations in the soil	Ikram et al. 1992
Greenhouse, sterilized soil	7.4 - 46.5	154 days (5,5 months)	not reported	*Glomus sp ., G. clarum,* ***G. intraradices****, G. macrocarpum, Acaulospora dilatata, A. spinosa, Gigaspora margarita, Entrophospora colombiana, Scutellospora calospora* (Foreign strains)	Ikram et al. 1993

Table 2. (continuation)

Growth chamber	70 days after planting: 10 112 days after planting :34 140 days after planting :73	140 days (5 months)	22 spores per g de soil	Mycelium of *Glomus clarum* that grew from *Pueraria phaseloides* inoculated roots	Ikram et al. 1994
Growth chamber, sterilized soil	45 days after inoculation: 0 90 days after inoculation: 10 135 days after inoculation: 30 180 days after inoculation: 47 225 days after inoculation: 47	225 days (≈ 9 months)	Not reported	*Glomus mosseae*	Schwob et al. 1998
Greenhouse, sterilized soil	18	192 days (6,8 months)	200 spores per pot	*Glomus clarum* (foreign)	Diniz 2007
Greenhouse, sterilized soil	Plantlets raised *in vitro*: 12.6 Plantlets raised in pots: 44.7	112 days (4 months)	200 spores per pot	Mixed inoculum of AM fungi (*Glomus* sp., *Acaulospora* sp., *Scutellospora* sp.)	Sosa-Rodriguez et al. 2009

The effect of the AMF inoculation on the nutrient contents of *H. brasiliensis* seedlings has been studied in controlled experiments. Ikram et al. (1992) tested a mixed AMF inoculum on rubber seedlings grown in the nursery (Table 2). The AMF treatment did not change significantly the nutrient contents in the leaves, and only a discrete augmentation in the content of P, K, Mg and Cu contents was found.

Ikram et al. (1994) studied the role of the mycelium network of *G. clarum* in the transfer of nutrients (P and N) from *Pueraria phaseloides* plants to rubber seedlings roots. They used a boxes-mesh system in which *H. brasiliensis* and *P. phaseloides* were grown in different compartments. Between the plant compartments, a central compartment was set up, and only the AMF mycelium was allowed to grow inside. The transfer of ^{15}N and ^{32}P from *P. phaseloides* to *H. brasiliensis* seedlings via the AMF mycelium was evaluated 20 weeks after the establishment of the systems, but no transfer between the plants was observed.

Nonetheless, the colonization of the *H. brasiliensis* plants (clone GT 1) by the mycelium that developed from the roots of *P. phaseloides* was successful and high colonization values were reported (Table 2). The shoot and root dry weights, and the N and P contents in the shoots of the *H. brasiliensis* plants colonized by the mycelium network were significantly higher as compared to the controls.

As the transfer of P and N from *P. phaseloides* to the rubber seedlings by the AMF mycelium was not demonstrated, Ikram et al. (1994) suggested that probably other indirect mechanisms (e.g. production of root exudates) could be influencing the association with the AMF. Moreover, it has been suggested that the absence of a direct effect on the accumulation of biomass or nutrients by the rubber seedlings indicates that AMF could be improving other features of the plants during the nursery stage, such as the resistance to water stress (Ikram et al. 1992).

Effects on plant physiology

Inoculation of *H. brasiliensis* seedlings with *G. clarum* has been shown to increase chlorophyll contents and transpiration rates. Improved concentration of soluble sugars and proteins in the leaves and roots, 192 days following AMF inoculation, was also reported (Diniz 2007). Similarly, seedlings inoculated with *G. mosseae* had higher transpiration rate, stomatal conductance and water-use efficiency, measured 9 months following inoculation. The effects on those parameters were related to the possible

capacity of the AMF to improve the performance of rubber plants under hydric stress conditions (Schwob et al. 1998).

Schwob et al. (2000) also found increased levels of root enzymatic activity (cinnamyl alcohol dehydrogenase, cell wall-bond peroxidases and some enzymes associated to root lignin synthesis), two weeks after the inoculation with *G. mosseae*. This activation was described as a transitory defence response elicited by the AMF strain (Schwob 1998).

Application of AMF to protect the rubber plants

Root colonization with AMF was associated with an increased resistance against the South American leaf blight *Microcyclus ulei*, which is considered as the main disease of *H. brasiliensis* in this region (Feldmann et al. 1990). The resistance of the rubber plants was evidenced by a decrease in both lesion size and sporulation of the pathogen in the AMF-colonized plants, suggesting that these fungi induced a resistance response in *H. brasiliensis*.

The impact of AMF colonization in the evolution of other rubber tree pathogens, as the root rot fungi *Rigidoporus lignosus* and *Phellinus noxius* is not known. Lignification, stimulation of cambium activity, wall thickening and callose deposition are frequent responses to the attack of these pathogens (Nicole 1984). Interestingly, the transient activation of lignification response in rubber roots after the colonization with the AMF *G. mosseae* has been described (Schwob et al. 2000). Thus, it could be possible that an early colonization with AMF stimulates a better root defence response of the roots against the root rot fungi.

AMF also can be used during the acclimatization of *in vitro* produced **plantlets as a mechanism of protection against abiotic stresses at the** transplantation (Kapoor et al. 2008). There are no previous studies about the effect of AMF on *H. brasiliensis* plantlets produced *in vitro*, but the improvement of the physiological status of rubber seedlings in the presence of AMF has been observed. Schwob et al. (1998) made nursery inoculations of *Glomus mosseae* on *H. brasiliensis* seedlings, and observed enhanced growth and increased photosynthesis, as well as improved water-use efficiency (i.e. increased transpiration and stomatal conductance), indicating that early AMF association could be useful to produce plantlets more resistant to water stress during their first years of growth in the field.

In vitro culture of arbuscular mycorrhizal fungi

In vitro culture of AMF allows the production of abundant and high-quality fungal material adequate for basic as well as applied studies (Fortin et al.

2005). In terms of biodiversity, root organ cultures (ROC) provide a tool for in-deep taxonomic analysis (i.e. through morphology and molecular biology), long-term propagation of contaminant-free biological material (Declerck et al. 2005), and also for physiological studies associating autotrophic *in vitro* produced plants with AMF (Voets et al. 2005; Dupré de Boulois et al. 2006; Voets et al. 2009).

In recent years, the *in vitro* autotrophic culture of plants associated to AMF has become an essential tool to study plant-fungus interactions (Voets et al. 2009; Gallou et al. 2010; de Jaeger et al. 2011; Koffi et al. 2012). This technology could also be used for the *in vitro* mass production of plantlets associated with AMF. For instance, the Laboratory of Mycology (UCL) successfully demonstrated that *in vitro* colonized banana plants had increased biomass during acclimatization in the greenhouse (Koffi et al. *Submitted*). Nevertheless, several parameters still have to be improved (e.g. mass production of inoculum, *in vitro* culture medium, *ex vitro* environmental conditions, type of *ex vitro* growth substrate…) in order to obtain procedures and inoculation protocols that can be easily integrated into commercial practices of mass plant micropropagation.

Rhizophagus irregularis MUCL 41833

The taxonomy of AMF was classically based on morphological traits of the spores, but this identification has shown to present limitations and low resolution. Molecular biological analyses of *Glomeromycota* have recently changed the general view of these fungi. In particular, some species as *Glomus intraradices* (one of the most intensely studied AMF) have been revised and changed (Stockinger et al. 2009). Nowadays, most of the ancient *Glomus intraradices* strains are recognized as part of the *Rhizophagus irregularis* species (Krüger et al. 2012).

Rhizophagus irregularis MUCL 41833 (Błaszk, Wubet, Renker & Buscot) C. Walker & Schuessler (2010) [as 'irregulare'] (Synonymy: *Glomus irregulare*) is an AMF strain widely used *in vitro*. The strain can be multiplied and maintained in ROC (Cranenbrouck et al. 2005) or autotrophic *in vitro* culture systems (i.e. HAM-P – Voets et al. 2005 or AM-P – Dupré de Boulois et al. 2006). Furthermore, this strain is perfectly adapted to growth in the MDP *in vitro* culture system, and has been successfully associated *in vitro* with several plants of economic importance, such as banana, potato, and more recently, oak tree (Dupré de Boulois et al. 2009; CESAMM Team, 2012; Prof. S. Declerck, *personal communication*).

Thus, for the methodological purposes of this research (i.e. to obtain a functional *in vitro* mycorrhization of *H. brasiliensis* plantlets), *R. irregularis* MUCL 41833 was an appropriate choice. The main reasons can be summarized as follows:

1. AMF are usually described as generalist microorganisms capable to establish a functional symbiosis with several plants, and different works suggest that there is no partner specificity (Smith and Read 2008).
2. *R. irregularis* (formerly *G. intraradices*) is present in natural and plantation soils of *H. brasiliensis* (Feldmann 1994).
3. *R. irregularis* has been successfully inoculated on *H. brasiliensis* plantlets on nursery conditions (Ikram et al. 1992; 1993).
4. *R. irregularis* represents a model fungus for the study of AMF (Stockinger et al. 2009).
5. *R. irregularis* MUCL 41833 is well adapted to the *in vitro* autotrophic culture in association with a host plant, being capable to colonize the roots of the host plant producing typical structures of a functional symbiosis (e.g. arbuscules) in a short time (Gallou et al. 2010).

Conversely, we cannot discard that AMF strains native of rubber soils (in natural stands or in plantations) could be more effective than the strain used during this research. Nevertheless, a screening of different AMF was out of the scope of this work (see the **Perspectives** section).

Research objectives

Micropropagation of *Hevea brasiliensis* is a source of selected plant material suitable to be used in genetic and/or physiological studies under highly controlled conditions, such as the interaction with AMF (Declerck et al. 2005). Furthermore, micropropagation techniques open the door for the mass production of selected rubber clones which have to be acclimatized in the nursery. At that moment, vigour and rapid growth recovery capacities are desired qualities for the plants that will be finally transplant to the field (Carron et al. 2009; Montoro et al. 2012).

AMF can improve the resistance of *in vitro* produced plants to potential transplantation stresses (Kapoor et al. 2008), and the improvement of growth, water-use efficiency and diminution of pathological status on rubber seedlings (rootstocks) and young trees have been described (Feldmann 1994; 2000; Ikram et al. 1993; Schwob et al. 1999; Sosa-Rodriguez et al. 2009). However, nothing is known about the effect of AMF on *in vitro* produced rubber plantlets, and the capacity of these microorganisms to improve the quality of micropropagated rubber material has not been considered before. Moreover, as no previous systems for autotrophic culture of *H. brasiliensis* exist, it was necessary to start by developing a suitable system of mycorrhization *in vitro*.

Therefore, the main objective of our thesis was to establish the impact of the *in vitro* culture conditions and the plantlet root growth on the association between the AMF *Rhizophagus irregularis* MUCL 41833 and *Hevea brasiliensis* clone PB 260 plantlets. To achieve this objective, different questions were addressed:

1. Is it possible to develop an autotrophic *in vitro* culture system adapted to the morphology of *H. brasiliensis* plantlets? Can we use such system to associate the *in vitro* cultured AMF *R. irregularis* MUCL 41833 to *H. brasiliensis* plantlets?
2. Is it possible to improve the root development/production of *H. brasiliensis* and its *in vitro* colonization by modifying the culture conditions (e.g. CO_2, IBA, MES, AC, taproot pruning treatments)?
3. Do *H. brasiliensis* root exudates have antifungal properties? Do these exudates impact the root colonization of *Medicago truncatula* plants by *R. irregularis*?

Chapter 1

Materials and methods: autotrophic *in vitro* culture systems to study the mycorrhization of the rubber tree

Preface

This section is largely based on the methodologies described in Cranenbrouck et al. (2005), Dupré de Boulois et al. (2009), and in the Manual of the International training on *in vitro* culture of Arbuscular Mycorrhizal Fungi (CESAMM Team 2012). These methods are currently applied to study the biology, physiology, morphology, phylogeny and genetics of the AMF (see Declerck et al. 2005). The micropropagation of plantlets without sugar in the culture medium and early inoculation with AMF presents several advantages such as the enhanced photosynthetic activity and the diminution of physiological and morphological disorders (Liu and Yang 2008). For this reason, the association of AMF to plants at the *in vitro* stage combined with the autotrophic micropropagation represents a decisive tool for mass production and commercial exploitation of AMF-colonized plants of economic importance. The production of *H. brasiliensis* under *in vitro* conditions is a potential market for this kind of biotechnology.

In this chapter, we described the adaptations that allowed the autotrophic *in vitro* culture of *H. brasiliensis* with AMF. Therefore, the *in vitro* cultivation methods on root organ culture (ROC; see Fortin et al. 2005 for a review) were not considered.

In vitro plant culture systems

Two major *in vitro* cultivation systems have been developed by the Laboratory of Mycology of the UCL for the *in vitro* association of AMF with autotrophic whole plants: (1) The half-closed arbuscular mycorrhizal-plant (HAM-P) *in vitro* culture system (Voets et al. 2005), in which the roots are maintained under strict *in vitro* culture conditions while the shoot develops in open air conditions and (2) the arbuscular mycorrhizal-plant (AM-P) *in vitro* culture system (Dupré de Boulois et al. 2006), in which both the AMF and the plant develop in a completely closed system. Both systems have been adapted to the growth of several plants (*M. truncatula, S. tuberosum*, *S. phureja*, *Plantago lanceolata*, *Centaurium erythraea, Gentiana verna, Oryza sativa, Physalis peruviana*, *Vitis berlandieri* Planch. x *Vitis rupestris* Scheele, *Nicotiana plumbaginifolia*, *N. tabaccum* and *Musa* spp.), with different AMF species such as *R. irregularis*, *R. clarus* and *Claroideoglomus claroideum* (CESAMM Team 2012).

Regardless of the system (HAM-P or AM-P), the AMF is associated with a 4 day-old *M. truncatula* seedling in the root compartment -RC (Figure 10) of a bi-compartmental Petri plate. Eight weeks following association, the AMF develops in the RC, crosses the partition wall (i.e. the plastic barrier which separates RC from HC), and spreads into the hyphal compartment -HC. This extraradical mycelium (ERM) network can be used for the production of thousands of spores, appropriate for later inoculation (see below).

Mycelium donor plant (MDP) *in vitro* culture system (adapted to the mycorrhization of *H. brasiliensis*)

The HAM-P *in vitro* culture system can also be used for the fast and homogenous mycorrhization of plants. The ERM network of actively growing hyphae (Figure 11 A) developed in the HC is able to colonize the plant roots in a short period of time (i.e. 5 to 7 days; Voets et al. 2009; Gallou et al. 2010). The system is then named mycelium donor plant (MDP) *in vitro* culture system, since an ERM network is used as inoculum instead of isolated spores.

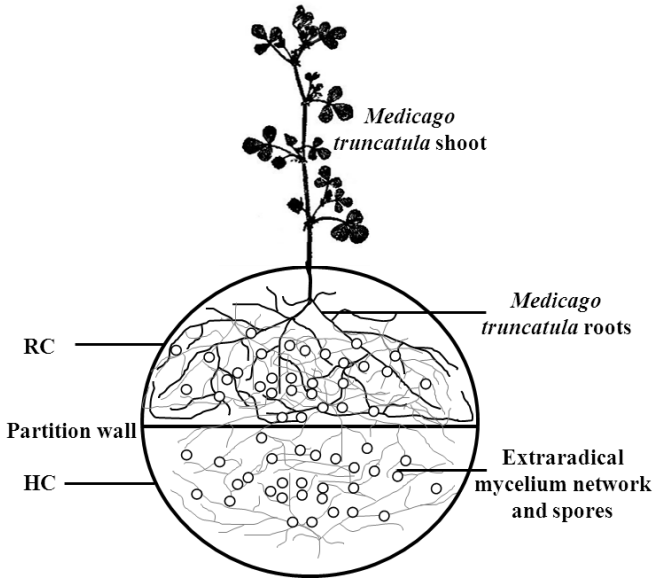


Figure 10. Schematic view of a half-closed mycorrhizal plant (HAM-P) *in vitro* culture system of *M. truncatula* inoculated with an AMF in the root compartment (RC). The system is established in a bi-compartmented Petri plate (90 mm diameter). The shoot of the plant remains outside the plate (See Voets et al. 2005 and 2009 for details). After 10 weeks of association, the extraradical mycelium network covers the hyphal compartment (HC), bearing thousands of spores (adapted from Voets et al. 2009).

The MDP *in vitro* culture system -originally developed for *M. truncatula* and *S. tuberosum* - has been successfully adapted to larger plants such as *Musa* spp. (Koffi et al. 2012) and *H. brasiliensis* (within this work). The system consists of at least 6 basic elements that are also present in the HAM-P *in vitro* culture system (Figure 10). Figure 11 shows a parallel between both systems. Each element is described below.

(1) The root compartment (RC): The lid of a 55 mm diameter Petri plate
(2) The hyphal compartment (HC): A 145 mm diameter Petri plate
(3) The AMF strain: e.g. *R. irregularis* MUCL 41833
(4) The culture medium: MSR without sucrose and vitamins + 3 $g.l^{-1}$ Phytagel™
(5) The donor plant: *M. truncatula* seedling
(6) The receiver plant: *H. brasiliensis* plantlet

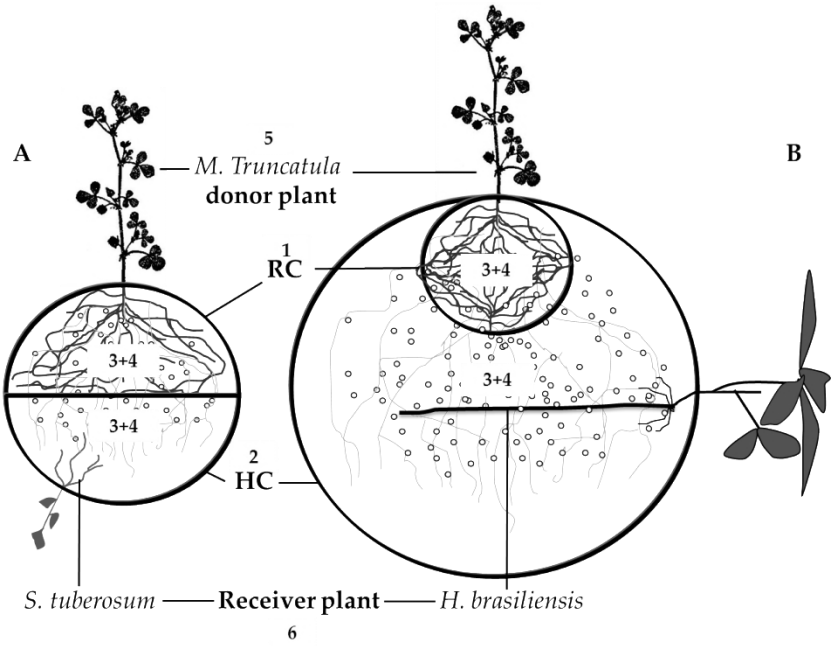


Figure 11. Schematic view of two mycelium donor plant (MDP) *in vitro* culture systems associating *M. truncatula* (5) with *R. irregularis* (3) in the root compartment -RC (1).

Eight weeks after inoculation, the extraradical mycelium densely covers the Hyphal compartment - HC (2). At that time, a small (e.g. potato; **A**) or large (e.g. rubber tree; B) receiver plant (6) is plated in the ERM for root colonization. **A.** Original HAM-P *in vitro* culture system (90 mm diameter). **B.** 145 mm diameter MDP *in vitro* culture system adapted to *H. brasiliensis* plantlets. Both compartments contained MSR medium (4) without sucrose and vitamins and solidified with 3 $g.l^{-1}$ Phytagel™ (Voets et al. 2009; Gallou et al. 2010; Sosa-Rodriguez et al. 2013a).

Construction of the Mycelium donor plant (MDP) *in vitro* culture system: root (1) and hyphal (2) compartments

The system consists of a polystyrene Petri plate (145 mm diameter x 20 mm height; Greiner bio-one®), in which the lid of a 55 mm diameter Petri plate is placed to physically separate the root compartment - RC (i.e. the 55 mm diameter Petri plate) from the hyphal compartment - HC (i.e. the 145 mm diameter Petri plate minus the 55 mm diameter Petri plate; Figures 11 B and 12). All the manipulations should be conducted under a laminar flow as follows.

1. Open the 55 mm diameter Petri plate plastic bag with a heated scalpel (to preserve the sterility in the bag).
2. Take only the cover with heat-sterilized long forceps. Put it inside the bottom of the 145 mm diameter Petri plate, next to the border of the Petri plate (as indicated in Figure 11 B).

The best way to work *in vitro* (especially if you have several units) in a clean and efficient mode is trying to make every step "**one at a time**" for all your replicates

3. Work in a vertical laminar flow with air extraction (to avoid inhalation of hazardous smoke during plastic melting). Use hot sterile forceps to melt the cover from the border to the top, making a long hole (See number 3 in Figure 12). Melt the bottom of the 145 mm diameter Petri plate making a small hole of approx. 2 mm diameter. The hole has to be next to the DC (i.e. 55 mm diameter cover), as shown in Figure 12. This hole allows the maintenance of the *M. truncatula* shoot outside the Petri plate and the roots inside the RC (Figure 11 [5]).
4. Make a second hole larger than the previous one (approx. 4 mm diameter) for the receiver plant *H. brasiliensis* (Figure 11 [6]) repeating the previous step. The second hole should be close enough to the RC to facilitate the rapid contact of the receiver plant with the ERM network of the AMF spreading in the HC (See Figures 11 B and 12 [4]).

Nutrients should be added weekly to the MDP *in vitro* culture system, starting from week 3 to the end of the experiment (see details in "System maintenance"). When working with small plants (e.g. *M. truncatula* and *S. tuberosum*), the systems could be completely opened to add fresh medium. Note that his procedure may increase the risk of contaminations. Alternatively, a tube with screw cap* can be glued to the cover of the MDP *in vitro* culture system (Figure 12). The tube is made by cutting the first 35-40 mm of a standard 50 ml centrifuge tube. To make a MDP *in vitro* culture system with a screw cap tube, follow the next steps.

*This tube was initially adapted for the MDP *in vitro* culture systems with banana (*Musa* spp.) as receiver plant (CESAMM Team 2012).

5. Work in a chemical hood to make the holes, and to cut and glue the different parts. Note that this manipulation cannot be made under sterile conditions. For this reason, after assembling of the tube with screw cap and the Petri plate covers, they must be sterilized at 25 kGy by gamma irradiation before using in the MDP *in vitro* culture systems.
6. Make a hole (approx. 15 mm diameter) in the cover of the Petri plate using a heated cork-borer. This hole has to be near to the RC, to facilitate the addition of MSR medium to both the RC and the HC compartments during the maintenance of the systems (Figure 12 [2]).
7. The cork-borer and scalpel are heated with the flame of a Bunsen burner to facilitate the cutting process. Cut the 50 ml centrifuge tube 40 mm away from the tube cap using the heated scalpel (alternatively, a saw could be used to go faster).
8. Glue the screw cap tube over the 15 mm hole of the cover, using plastic thermo-fusible glue adapted to plastic glueing (Figure 12 [2]).

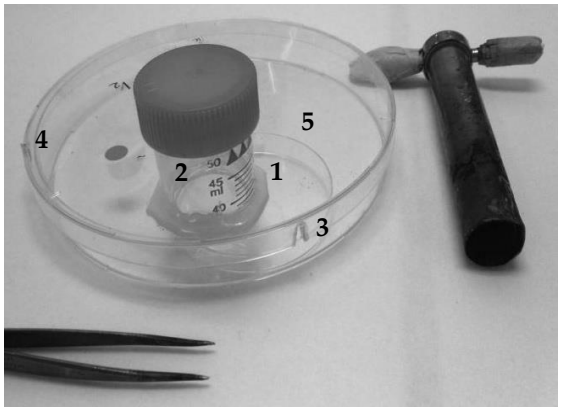


Figure 12. 145 mm MDP *in vitro* culture system cover assembled with a screw cap tube for system maintenance.
(1) The 55 mm diameter Petri plate that separates the root compartment -RC from the hyphal compartment -HC (5); (2) screw cap tube glued for systems maintenance; (3) hole for the *M. truncatula* donor plant shoot and (4) hole for the *H. brasiliensis* receiver plant shoot.

Inoculation of the AMF (3), addition of the culture medium (4) and placing the donor plant (5)

Rhizophagus irregularis (Błaszk, Wubet, Renker & Buscot) C. Walker & Schuessler (2010) [as 'irregulare'] MUCL 41833 (Synonymy: *Glomus irregulare)* is an AMF strain widely used *in vitro*. The strain can be multiplied

and maintained in ROC (Cranenbrouck et al. 2005) or autotrophic *in vitro* culture systems (i.e. HAM-P – Voets et al. 2005 or AM-P – Dupré de Boulois et al. 2006). For mass production in the HAM-P *in vitro* culture system, the AMF is associated with a 4 day-old *M. truncatula* seedling in the root compartment - RC (Figure 10 and Figure 11 A) of a bi-compartmented 90 mm diameter Petri plate (Voets et al. 2009). After 10 weeks, the ERM network totally covers the hyphal compartment - HC, bearing thousands of spores that can be used as inoculum following the next steps.

The inoculation with a plug of gelled medium containing spores is possible for AMF producing large densities of spores (e.g. for *R. irregularis*, a 5 mm diameter plug of medium can contain 100-150 spores)

1. Select a 2 to 4 month-old culture presenting numerous spores in the HC. Check carefully under stereo-microscope that no contaminants are present, and use a marker to select the areas of the HC presenting high density of spores.
2. Work under a laminar air flow. Cut several pieces of gel using a 5 mm diameter sterile cork-borer.
3. Extract a piece of gelled medium and transfer it to the middle of an empty RC using a sterile scalpel. This procedure can be done in all the systems before the next step.
4. After placing the plug of gel in the RC, the MSR medium* can be poured.
5. Pour the culture medium at 45°C. First, pour the medium (20 to 25 ml) into the RC (to avoid flotation and displacing of the 55 mm diameter Petri plate that separates the RC). Then, pour 100 to 125 ml in HC.

*The composition of the Modified Strullu-Romand (MSR) culture medium in described in Cranenbrouck et al. (2005) Add the medium to the Petri plates as described below. Note that the DEV3 culture medium (used for the embryo germination during the *H. brasiliensis* somatic embryogenesis; see Lardet et al. 2007 for details) cannot be used because the AMF is not able to growth in a medium with high nutrient content.

Different substances (i.e. treatments) can be added during the culture medium preparation. For instance, 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) buffer was used in the experiments described in Chapter 2, and activated charcoal (AC) was used during the experiments described on Chapter 3

6. *M. truncatula* Gaertn. Cv. Jemalong A17 clone (SARDI Genetic Resource Centre, Australia) seeds are disinfected by immersion in a solution of sodium hypochlorite (8°chl = 2.5 $g.l^{-1}$ of active Cl) for 15 min and rinsed 3 times in deionized sterile water. They are subsequently germinated in Petri plates filled with MSR medium without sucrose and vitamins, solidified with 3 to 3.5 g l^{-1} Phytagel™. The seeds are ready for use after 4 days of germination in the dark at 20°C (CESAMM Team 2012).

Using MSR medium **with** sucrose allows you an early detection of potential contaminations in the seeds inner layer

7. One 4 day-old *M. truncatula* seedling presenting vigorous growth and a strong primary root, is plated in the RC, with the root close to the spores plug, and the shoot extending outside the Petri plate via the hole (Figure 12 [3]).

(6) The receiver plant: *Hevea brasiliensis* plantlet

Clones of *H. brasiliensis* clone PB 260 (Prang Besar, Malaysia) are produced in CPN Bio-Michelin/Ladoux (France) by somatic embryogenesis. In the last part of the process (detailed in Lardet et al. 2007, the embryos are germinated in glass tubes (25 mm diameter x 200 mm length; Figure 13) filled with 30 ml of DEV3 culture medium, an agar gelled culture medium adapted to the embryo germination and *in vitro* growth of *H. brasiliensis*. This mixotrophic culture medium contains 80 $g.l^{-1}$ sucrose without growth

regulators. The plantlets develop in a growth chamber set at 27 °C, with a 12 hd^{-1} photoperiod under a photosynthetic photon flux of 60 µmol m^{-2} s^{-1}.

Only plantlets with 1-3 leaves, 40 to 60 mm stem height, 40 to 60 mm taproot length and fully developed secondary roots (5-10 lateral roots of 1-3 cm length) should be used.

Figure 13. *H. brasiliensis* clone PB 260 (Prang Besar, Malaysia) plantlets.

1. Use long sterile forceps (at least 20 cm long) to transfer the plantlet from the tube to the MDP *in vitro* culture system. The taproot is placed on the surface of the medium in the HC, and the shoot extending outside the Petri plate via the hole (Figure 12 [4]).

To avoid contamination, flame the tube containing the *H. brasiliensis* plantlets before the transfer to the MDP *in vitro* culture system. It is also important to be sure that after the transfer to the RC, most of the root system is placed inside the culture media and not only on the surface.

2. Carefully close the Petri plate by placing the cover above the plants shoots.
3. Seal the systems with a strip of Parafilm™ (Pechiney, Plastic Packaging, Chicago, IL 60631). Then, put silicon grease (N. 6674.0050, VWR® BDH® PROLABO®) around the shoot of the two plants, as described by Voets et al. (2009) for the HAM-P system.
4. Add a piece of white adhesive re-usable material around the *H. brasiliensis* shoot (Figure 14). This material can be sterilized in an

autoclave at 121°C for 15 min. It is known as "Blu-Tack"®-White (Bostik®) or "Tack-it"® (Faber-Castell®) in UK, or Patafix® (UHU®) in Belgium and France.

5. Finally, seal the system with plastic wrap (2 cm-large roll; Figure 14).

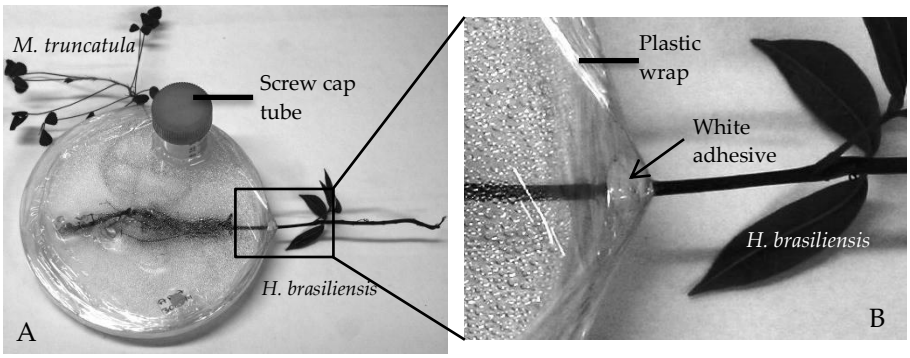


Figure 14. Details of the seal in the *H. brasiliensis* MDP *in vitro* culture system.
A. General view of the sealed system; **B.** Close view of the piece of white adhesive re-usable material (arrow) around the *H. brasiliensis* shoot.

6. Cover the Petri plate with an black plastic bag, to keep the roots and the AMF in the dark, and incubate the culture system horizontally in a growth chamber under controlled conditions: a constant relative humidity of 90%, 12 hd^{-1} photoperiod under a photosynthetic photon flux of 30 to 60 µmol m^{-2} s^{-1} from week 1 to 4 (Lardet et al. 2007) and 200 to 250 µmol m^{-2} s^{-1} starting from week 5 until the end of the experiment.
7. Set temperature at 26°C during the day and 23°C during the night.

There are to possibilities (1) First place the donor plant in the RC, and 8 weeks later, place the *H. brasiliensis* in the HC (i.e. when the ERM network spread into HC (Chapters 2 and 3), or (2) place the donor plant and the *H. brasiliensis* plantlet at the same time (Chapter 2). Make (2) if you need a "root production stage" prior to the contact with the mycelium network.

System maintenance

Since the shoots of the plants develop under open-air conditions, transpiration leads to rapid depletion of the medium (especially if relative humidity is lower than 70%). Moreover, the addition of medium is not only necessary to avoid desiccation, but also to maintain an adequate level of nutrients for the growth of the plants and the AMF.

If you are working with a 90 mm or a 145 mm diameter system without screw cap tube:

1. Remove the Parafilm™, make sure the shoot of the plants are kept in place and does not touch the internal surfaces of the Petri plate, and open the plate.
2. Add MSR medium without sucrose and vitamins at 45°C in both compartments. Graduated tubes (i.e. sterile 50 ml centrifuge tubes) can be used.
3. Carefully close the Petri and seal it with a strip of Parafilm™. The holes around the plant shoots are then carefully plastered with silicon grease to avoid contamination.

If you are working with the modified system (Figure 14 A):

a. Remove the cap of the tube. Keep it in a clean place.
b. Add medium at 45°C in both compartments. Graduated tubes (i.e. sterile 50 ml centrifuge tubes) can be used.
c. Close the tube.

With a good seal, the "screw cap tube" system can almost eliminate the risk of contamination during the manipulation of the systems for several weeks. Nevertheless, the sterilization with gamma irradiation (that is expensive) of the systems covers modified with the screw cap tube before using them to assemble the systems is mandatory.

A faster alternative

Alternatively to the screw cap tube, the hole made in the cover of the Petri plate can be covered with a three-layers piece of 3M™ Micropore™ medical tape of about 2 cm^2 (Figure 15). This material is porous (allows gas exchange) but keeps sterility and is also entirely autoclavable. You can then replace it every week during the system maintenance, following the MSR medium addition. In this case, use sterile 20 ml pipettes or syringes to add the medium.

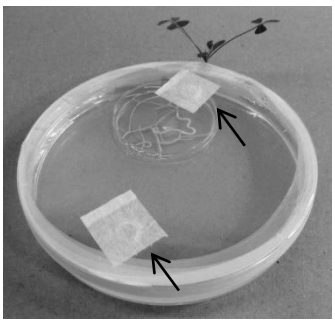


Figure 15. Detail of the holes used to add the MSR medium, covered with a piece of 3M™ Micropore™ medical tape (arrows). The sterilization of this material by autoclave (15 min. at 121°C) is possible.

If working at 90% of relative humidity, the level of the medium remains constant, but not the level of nutrients. **Add 1 ml of liquid 10x concentrated MSR medium*** instead of 10 ml of the normal gelled one

*Liquid medium will spread from the surface to the rest of the solid medium in the Petri plates. It can be added using a 1000 µl pipette and sterile tips (see Dupré de Boulois et al. 2006 for details).

The overall process for the *in vitro mycorrhization* of *H. brasiliensis* plantlets using a MDP *in vitro* culture system is summarized in Figure 16.

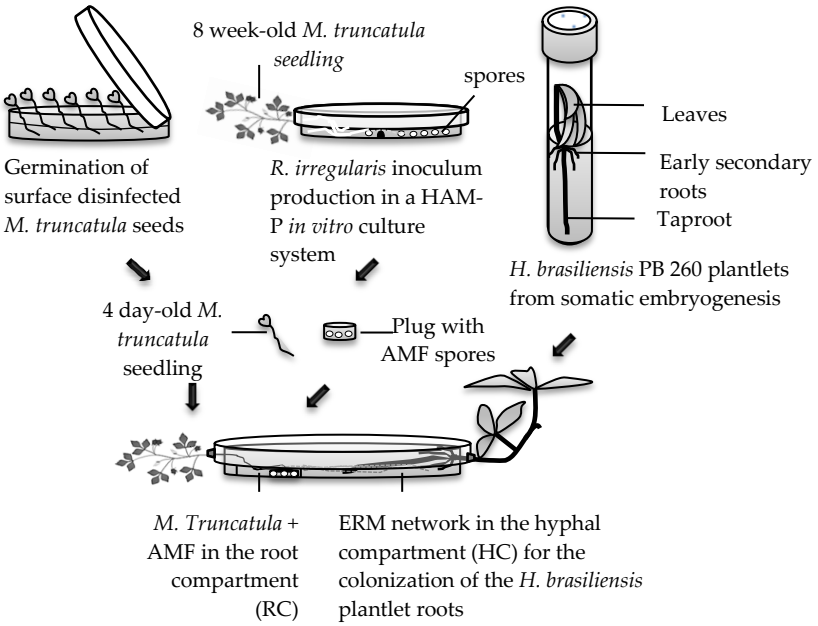


Figure 16. Schematic representation of the autotrophic mycelium donor plant *in vitro* culture system adapted for the mycorrhization of rubber tree plantlets.

The AM-P *in vitro* culture system (Dupré de Boulois et al. 2006) can also be adapted to large plants as *Musa* spp. (Koffi et al. 2009) or *H. brasiliensis* (our work). Using this system the risk of contamination is reduced, as whole plant (shoots and roots) grows *in vitro* inside a closed system (Figure 18).

AM-P *in vitro* culture system (adapted to the mycorrhization of *H. brasiliensis*)

This system (Figure 18) is particularly useful to obtain the *in vitro* mycorrhization of the plantlets using spores instead of an ERM network. It can be easily adapted to large plants as *Musa* spp. (Koffi et al. 2009) and *H. brasiliensis*. For assembling the system it is possible to follow the methodology explained by Dupré de Boulois et al. (2006). The main adaptations are the use of a 145 mm diameter Petri plate as RC, and the plastic bag as shoot compartment -SC (see below).

1. Replace the 50 cm centrifuge tube cap used in Dupré de Boulois et al. (2006) by a plastic sun bag with 6 ventilation strips (Full GasMicrosac with filters 0.22 μm; SAOC Microsac, Eke, Belgium).
2. The size of the plastic bag is obtained by cutting and sealing with a bag sealer.
3. Make the junction between the tube and the bag with cellophane plastic wrap.
4. Sterilize the systems at 25 kGy by gamma irradiation before utilization.
5. Fill the root compartment (RC) with MSR medium lacking sucrose and vitamins.
6. Inoculate the Petri plate with plugs containing mycelium and spores (see the recommended position of the plugs in Figure 17). Note that placing plugs around the early secondary roots (ESR) is not useful because the growth and colonization by the AMF of these roots are limited (see Chapter 2).
7. Put one *H. brasiliensis* plantlet on the surface of the Petri plate. To manipulate the plantlet, proceed as explained in the previous section.
8. Once the root system is placed on the medium, introduce *carefully* the shoot of the plantlet into the hole of the cover using sterile forceps.
9. Close the system using plastic wrap, and place it in the same culture conditions as described above, except for the relative humidity (RH): Since the sun bag is protecting the shoot of excessive transpiration-desiccation, a RH level of 70% can be maintained in the growth chamber.
10. Each Petri plate is covered with a black plastic bag to maintain the root system in the dark.
11. Maintenance: depletion of the medium level will be low. Use the same methodology as explained for the modified HAM-P *in vitro* culture system (i.e. addition of x10 concentrated MSR medium).

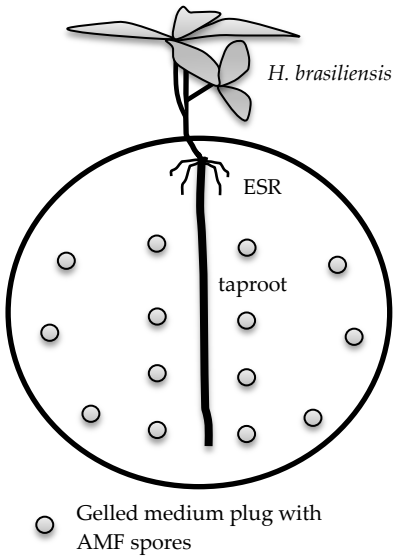


Figure 17. Suggested inoculation points of the AM-P autotrophic *in vitro* culture system (Schematic view of the RC, without the cover) adapted to the mycorrhization of *H. brasiliensis*.
Every single plug can contain 15-20 spores. The plugs of spores come from a HAM-P *in vitro* culture system used for the AMF inoculum production (see previous section). Put the plugs near to the taproot (early secondary roots – ESR has limited growth).

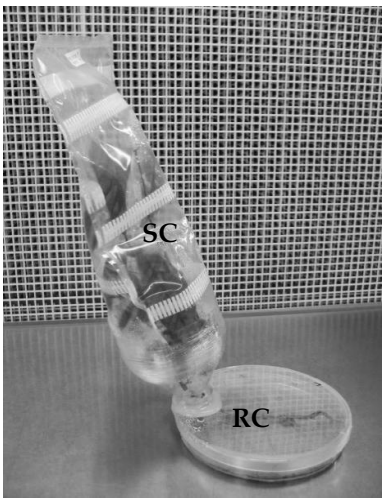


Figure 18. The AM-P autotrophic culture system adapted to the *in vitro* colonization of *Hevea brasiliensis* plantlets. Shoot compartment (SC); root compartment (RC).

It is important to highlight that compared with other autotrophic *in vitro* mycorrhized plants (e.g. *M. truncatula*), the *in vitro* root development of *H. brasiliensis* is slow (see on next chapter for details) and the mycorrhization with spore plugs used in this system will take several (15-20) weeks. Consequently, mycorrhization with a mycelium network (instead of inoculation with spores) is highly recommended.

Clearing and staining of *H. brasiliensis* roots

H. brasiliensis has thick and pigmented roots. The technique of Phillips & Hayman (1970) for staining was therefore slightly adapted.

1. Sample and dry the roots completely before storage if the staining is not done immediately. Avoid storing the roots in aqueous solutions.
2. Use Figure 19 to select and sort the roots before staining.
3. Cut the larger roots (more than 10 cm long) in shorter fragments.
4. Place the sorted roots in different 15 ml centrifuge tubes.
5. If the roots are young (SRAS, LSR, TR, taproot apex A) and white, clear them in 10% KOH at 90°C for 1 h.
6. If the roots are old and pigmented (taproot, EST), clear in 10% KOH at 90°C for 2 h and then, leave them overnight at room temperature.
7. Wash the roots with fresh 10% KOH.
8. Clear with H_2O_2 (3%) (5 min, or until bleached) to remove tannins.
9. Rinse thoroughly.
10. Stain with blue ink vinegar solution (5% acetic acid (household white vinegar) with 5% blue ink (Parker) according to Vierheilig et al. (1998) at 90°C for 3-5 min.
11. Remove the excess of stain in clean lactoglycerol (v:v:v - 1:2:1 - lactic acid:glycerol:H_2O).

AM fungi roots colonization measurement

We used the "magnified intersections method" developed by McGonigle et al. (1990) to assess the root colonization.

1. Transfer the roots from the centrifuge tube to a 90 mm diameter Petri plate to facilitate the manipulation.
2. Cut the stained roots in fragments of 1 – 4 cm using a scalpel with a new blade.

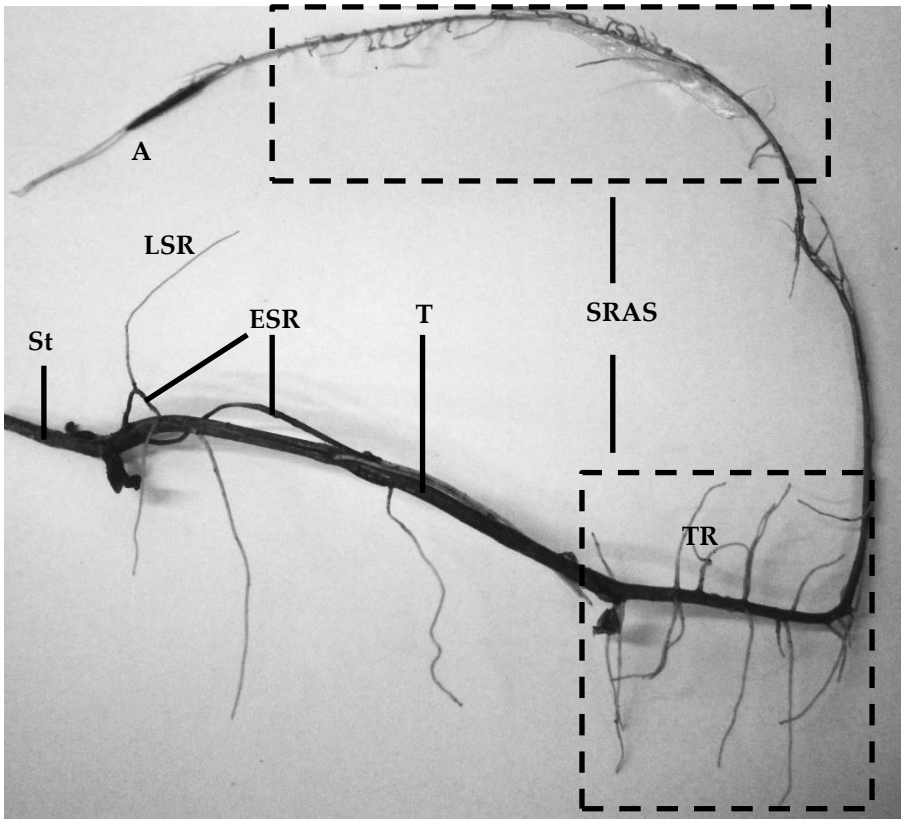


Figure 19. Different types of roots produced by *H. brasiliensis* plantlets *in vitro.* (St) Shoot, (A) main apex, (T) taproot, (ESR) early secondary roots, (SRAS) secondary roots of the acropetal sequence, (LSR) late secondary roots, (TR) tertiary roots. Roots named according to Le Roux and Pagès (1996).

1. Use the same lactoglycerol solution used at the end of the staining process as mounting medium. Place some strips of this solution on the slide with a Pasteur pipette.
2. Use forceps to take the roots and align them in parallel to the long axis of the slide.
3. Carefully place the coverslip.
4. Place the slide under the microscope (compound bright-field light microscope).
5. Use 10x ocular magnification.
6. Use the 4-10x objectives to have an overview of the roots. Then, place the microscope field at the beginning of the slide.
7. Use the 20-40x objectives to assess the root colonization.

Move the field using the specimen holder. It is recommended to make 10 complete passes across each slide perpendicular to its axis. The distance between the passes is not critical, but should be constant. At least 150 fields should be measured (Figure 20 A).

Quantification

1. Use a manual tally counter with two places to register (Figure 20 B). Name one of them as the "positive-intersection counter" (+) or the "total number of intersection counter", and the second one, as the "negative-intersection counter" (-).
2. Use a format similar to the table showed in Figure 20 C to register the presence of each structure.
3. At each intersection between the root and the vertical eyepiece cross-hair (Figure 19 D), a mark is made (i.e. the "positive-intersection counter" is augmented by one) if it crosses an arbuscule, vesicle or hyphae.
4. At the same time, the presence of each structure is registered in the table in the following categories (Figure 20 C): "arbuscules" (Figure 20 D 3); "hyphae only" (Figure 20 D 2) and "vesicles" (Figure 20 D 1).
5. Even if the cross-hair crosses more than one structure (arbuscule, vesicle or hyphae), each category is incremented only by one.
6. If no AMF is observed in the root (Figure 20 D 4), the "negative-intersection counter" (-) is incremented by one.
7. When arbuscules, vesicles and hyphae are visualized at the same time in the same intersection, only the first two categories are incremented by one (do not increment the hyphae category), and the total number of intersections is only incremented by one. The colonization by structure (arbuscule, vesicle) is calculated following the equation 1:

(1) *% of colonization* = (Count of the particular structure/total number of intersections examined)*100*

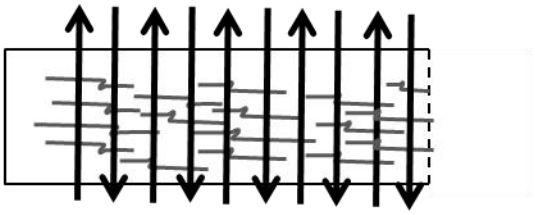
*Arbuscular or Vesicular.

The total root colonization (also called positive or hyphal colonization) is calculated as the proportion of non-negative intersections (equation 2):

(2) *% of total root colonization = ((total number of intersections examined – number of negative intersections)/total number of intersections examined)*100*

8. The observation of 100 to 150 intersections per sample is recommended.

A



B

C

Sample	Slide No.	Negative (-)	arbuscule	vesicle	hyphae only	Total counts (+)

D

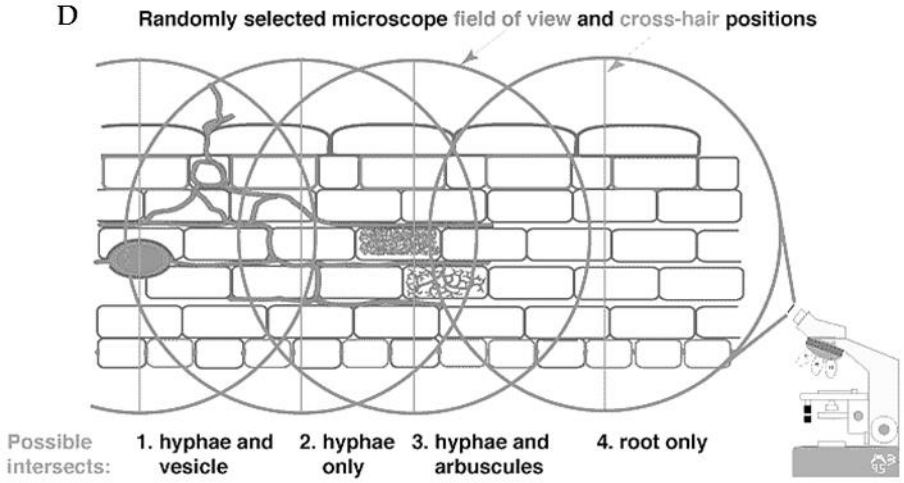


Figure 20. Diagram of the root colonization quantification.

A. Root fragments disposition on the slide. The arrows are showing the direction of the passes across the slide. Every intersection between the microscope field and the roots is examined. **B.** Manual tally counter with two places to register (+ and -). **C.** Example of a table to register the intersection by structures. **D.** Possible intersection points between the microscope crosshair and the root to quantify AMF structures (McGonigle et al. 1990; Brundrett et al. 1996).

Rhizophagus irregularis physiology: SDH enzymatic activity

Vital staining (SDH, ALP and ACP enzymatic activities) was applied in Chapters 3 and 4. The activity of these specific enzymes was used for the visualization of the metabolically active fungal tissue in the ERM network of the AMF (Vierheilig et al. 2005).

The succinate dehydrogenase (EC 1.3.99.1) -SDH enzymatic activity is used as an index of fungal metabolic activity because it acts in the tricarboxylic acid (TCA) cycle. SDH quantification can be used as an indicator of the respiratory fungal activity (Saito 1995).

1. Prepare the stock solutions (they can be kept at 4°C for approx. one month):
   - 0.2 M Tris/HCl pH 7.4
   - 5.0 mM $MgCl_2$
   - 1.0 M sodium succinate

2. From the stock solutions, take the following quantities to make the working solution in a 15 ml centrifuge tube:
   - 2.5 ml Tris/HCl
   - 1.0 ml $MgCl_2$
   - 2.5 ml sodium succinate
   - 4.0 ml distilled water

3. Agitate the working solution and add 10 mg of Nitrotetrazolium blue chloride (NBT).

4. For an easy manipulation and staining of several samples at the same time, we recommend the use of a 96 Well Microplate of 128 length x 85.5 width (Greiner Bio-one®; Figure 21 B). Use columns (named 1 to 12) for the samples and the first 7 rows (named A to G) for the different staining solutions, as follow:
   A. Distilled Water (W1)
   B. Distilled Water (W2)
   C. Distilled Water (W3)
   D. SDH working solution (contains NBT) (S)
   E. Distilled Water (W4)
   F. Acid Fuchsin solution (0.1% acid fuchsin in lactic acid) (F)
   G. Lactoglycerol (lactic acid:glycerol:water; 1:2:1 v:v:v) (L)

5. Use an adjustable micropipette and disposable tips to add 200 µl of solution in each well (change the tip between each solution).
6. Prepare buffer sodium citrate (0.01 M; pH 6) by adding 0.9 ml of a 0.1 M solution of citric acid and 4.1 ml of a 0.1 M solution of sodium citrate in 50 ml of distilled water (Doner and Bécard 1991).
7. Use a scalpel to take samples of the medium from the HC. Take samples of the same size (e.g. 1 cm x 1 cm squares) and make sure that the development of the mycelium is the same within a treatment and if possible, also between treatments. If this is not possible between treatments, take samples from the same area in the HC.
8. Place each sample in a 90 mm diameter Petri plate. Cut the gel piece with a scalpel to obtain very small pieces, to facilitate the contact with the sodium citrate buffer and the dissolution of the gel. Add 20 ml of citrate buffer to each sample. Agitate the Petri plate slowly on a rotating agitator (50 rpm) at room temperature for approx. 30 min.
9. When the gel is dissolved, use fine tip forceps to take the sample of the AMF mycelium.
10. Start the staining by washing the samples (to remove residues of citrate buffer or MSR medium) by transferring them from the first well (W1; see Figure 21 B) of the Microplate, and then transfer to the second and third well (W2 and W3).
11. Transfer the samples to the fourth well that contains the working solution (called S in the Figure 21 B). Incubate the samples overnight in the dark.
12. Wash the samples (i.e. transfer them to the W4) during 15 min.
13. Counterstain the samples in acid fuchsin solution for 15 min – 1 h (F).
14. Transfer the samples to the lactoglycerol solution (L) to remove the excess of acid fuchsin.

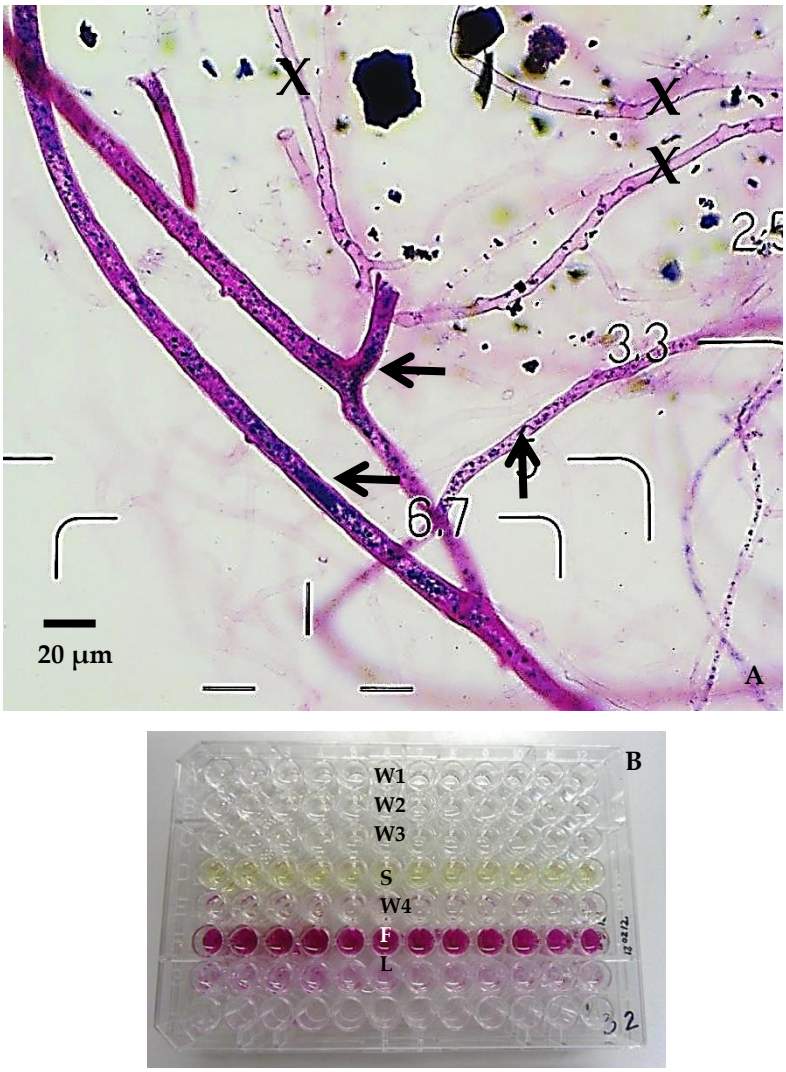


Figure 21. SDH enzymatic activity cytochemical staining.

A. Extraradical mycelium of *Rhizophagus irregularis* MUCL 41833 collected after gel dissolution from the hyphal compartment of a MDP *in vitro* culture system. The mycelium was stained with nitrotetrazolium blue chloride and counterstained with acid fuchsin to reveal the SDH enzymatic activity (black arrows: dark precipitates inside the hyphae). X represent empty hyphae or hyphae without activity. **B.** Example of 96 Well Microplate (Greiner Bio-one®) used during for the successive transfers of the mycelium to the different staining solutions.

Rhizophagus irregularis physiology: Phosphatase enzymatic activity

Phosphatases are involved in the hydrolysis of organic phosphorus (P) compounds (e.g. P esters) into inorganic phosphorus. In AMF, these enzymes are involved in the fungal P metabolism, i.e. fungal nutrition and the intracellular P translocation. Alkaline phosphatase (EC 3.1.3.1) -ALP activity in the extraradical mycelium has been associated with the P uptake from the rhizosphere. Acid phosphatase (EC 3.1.3.2) -ACP activity in the intraradical mycelium has been associated with the transfer of P from the mycelium to the host plant (Joner et al. 2000; van Aarle and Olsson 2008).

1. For the acid phosphatase, prepare the following stock solutions:

   - Acetate buffer 200 mM, pH 5.5:
     a) 0.2 M acetic acid (for 200 ml, add 2.402 ml of glacial acetic acid and complete the volume with dH_2O)
     b) 0.2 M Na Acetate (for 200 ml, add 3.292 g Na-acetate and complete the volume with dH_2O)

     Gently, add the "a" (acetic acid) solution to the "b" (Na Acetate) solution, checking the pH. (i.e., use the acetic acid solution to obtain the 5.5 pH).

2. For the alkaline phosphatase, prepare the following stock solution:

   - Tris HCl buffer 0.2 M, pH 8.5: For 200 ml, add 4.8456 g Tris , adjust pH with HCl 0.1 M, and complete the volume with dH_2O

3. For each enzyme, take the following quantities of the stock solutions, to make the working solution in a 15 ml centrifuge tube:
   A. 5.0 ml of buffer (Acetate or Tris) + 11 mg (1.1 mg/ml) of alpha-naphthyl phosphate Na salt
   B. 5.0 ml of dH_2O + 10 mg (1.0 mg/ml) of Fast blue RR
4. Mix A and B just before use.
5. Repeat the steps 4 to 10 of the previous section. In step 11, use the working solution for the phosphatases (ACP-Acetate; ALP -Tris buffer). Incubate the samples for 1:30 h min at room temperature in the dark. Repeat steps 12 to 14 of the previous section.

During this work, we only took extraradical mycelium from the HC of each MDP *in vitro* system, but the enzymatic activity can also be measured using these cytochemical techniques for the intraradical mycelium, adding a digestion step of the root tissues (see Saito 1995; van Aarle et al. 2002b; Zocco et al. 2011)

Quantification

The methodology published by McGonigle (1990) can be used to assess the enzymatic activity of the mycelium. For the microscopic observations, the samples of mycelium can be mounted on microscope slides with lactoglycerol. The formazan (for SDH) or Fast blue RR granular precipitation (for ALP and ACP) is assessed with a compound bright-field light microscope at x500 magnification (Figure 21 A).

1. Use a manual tally counter with two places to register (Figure 20 B). Name one of them as the "total-intersection counter", and the second one, the "active hyphae-intersection counter".
2. At each intersection between one hypha and the vertical eyepiece cross-hair, a mark is made (i.e. the "total-intersection counter" is augmented by one)
3. If it crosses a granular precipitate zone of the hypha (Figure 21 A, black arrows), the "active hyphae-intersection counter" is incremented by one.
4. If no dark precipitate is observed in the hypha (Figure 21 A, X marks) only the total intersection-counter is incremented.
5. The percentage of active hyphae is calculated following the equation 3.

(3) *% of active hyphae*= (Count of active hyphae/total number of intersections examined)*100*

*SDH, ALP or ACP. The observation of 100 to 150 intersections per sample is recommended.

Chapter 2

In vitro mycorrhization of the rubber tree *Hevea brasiliensis* Müll. Arg.

Preface

In this chapter, we adapted the mycelium donor plant (MDP) *in vitro* culture system developed by Voets et al. (2009) (see description in **Chapter 1**) to associate *H. brasiliensis* plantlets with the extraradical mycelium (ERM) network of the AMF *R. irregularis*. After preliminary *in vitro* assays, only traces of root colonization were observed after several weeks of contact with the ERM. Therefore, three factors were tested to stimulate the root development and subsequent colonization by the AMF: (1) high CO_2 content in the growth chamber atmosphere; (2) hormonal stimulation of the root growth with Indole-3-butyric acid - IBA) and (3) buffering of the culture medium with 2-(N-Morpholino)ethanesulfonic acid sodium salt -MES. The beneficial effects of these factors have been reported on the *in vitro* growth of several other *in vitro* raised plants (Pospísilová et al. 2007) but have never been tested on *H. brasiliensis* plantlets.

In vitro mycorrhization of the rubber tree *Hevea brasiliensis* Müll. Arg.

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Abstract

In vitro cultivation systems of arbuscular mycorrhizal fungi are useful tools to study the interaction between plants and their fungal symbiont, but also to develop new biotechnologies. Plantlets of the latex-producing species *Hevea brasiliensis* clone PB 260 were grown in a dense extraradical mycelium network of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 developed from a mycelium donor plant (*Medicago truncatula* A17). The factors indole-3-butyric acid (IBA), 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) buffer, and carbon dioxide (CO_2) were tested on root development and colonization by the fungus. No colonization was observed in the presence of plantlets pre-treated with IBA. The highest levels of root colonization were obtained when plantlets were mycorrhized under a high CO_2 concentration (1000 μmol mol^{-1}) with MES (10 mM) added to the growth medium. Widespread root colonization (with presence of arbuscules, intraradical mycelium, and intraradical spores/vesicles) was predominantly observed in newly produced roots. Therefore, it appears essential to improve root initiation and growth for improving *in vitro* mycorrhization of *H. brasiliensis*. We demonstrated the potential of the "mycelium donor plant" *in vitro* culture system to produce colonized *H. brasiliensis* plantlets before their transfer to *ex vitro* conditions.

Keywords Natural rubber • Arbuscular mycorrhizal fungi • Mycelium network • Autotrophy • *In vitro* culture

Introduction

Hevea brasiliensis Müll. Arg. is the tree species exploited for the production of natural rubber (Compagnon and D'Auzac 1986). The demand for this raw material is steadily increasing because it has particular properties of elasticity, stiffness, and resistance to heating that are superior to synthetic rubber (Delabarre and Serier 1995). Therefore, this tree has an undeniable economic importance for the countries of production.

Genetic engineering of *H. brasiliensis* and massive clonal propagation of selected genotypes are two of the most important approaches to obtain trees with improved latex production (Leclercq et al. 2012; Montoro et al. 2012). For instance, *in vitro* culture by somatic embryogenesis was developed for the production of high quality, healthy, and uniform plants, which are later acclimatizated in the nursery. Rapid growth recovery and high vigour are required qualities of the material before the final transplantation to the productive fields (Carron et al. 1995; Lardet et al. 2007; Carron et al. 2009; Montoro et al. 2012).

Several *in vitro* culture conditions have been tested on various plant species to improve their performance during the acclimatization (see Hazarika 2006 for a comprehensive review). The increase in carbon dioxide (CO_2) to stimulate photosynthesis (Buddendorf-Joosten and Woltering 1994), the regulation of pH in the medium (Harbage and Stimart 1996), and the development of autotrophic culture systems (i.e., under high light intensity and limited or absence of sugar in the medium) have been attempted to minimize the problems during the acclimatization (Fujiwara and Kozai 1995; Jeong et al. 1995). Root initiation and quality of the rooting system *in vitro* also have important consequences on plant quality after transplantation to *ex vitro* conditions and different *in vitro* rooting methods (e.g., application of plant growth regulators) have been developed (McClelland et al. 1990; Díaz-Pérez et al. 1995).

The association under *in vitro* conditions with plant-beneficial microorganisms, such as plant growth promoting rhizobia and mycorrhizal fungi is another option that has been suggested to increase growth and resistance to environmental stresses (e.g. water stress or pathogens attack) during the *ex vitro* acclimatization phase (Martins et al. 1996; Nowak 1998; Hazarika 2006; Liu and Yang 2008). The *in vitro* association of trees with ectomycorrhizal fungi was shown to improve rooting, biomass production, and acclimatization to *ex vitro* conditions (Martins et al. 1996; Díez et al. 2000). Banana plants associated *in vitro* to arbuscular mycorrhizal fungi

(AMF) showed increased biomass during the hardening phase in the greenhouse (Koffi *Personal communication*). Propagating plants under *in vitro* autotrophic culture conditions in association with their root symbionts (e.g. AMF) may thus represent an alternative to improve acclimatization of plants to *ex vitro* conditions, and possibly to accelerate transplantation to the field.

Recently, Voets et al. (2009) developed a Mycelium Donor Plant (MDP) *in vitro* culture system that allowed the fast and homogenous colonization of various plantlets such as barrel medic (*M. truncatula*), banana (*Musa acuminata* L., AAA genome, cv. Grande Naine; Koffi et al. 2012), and potato (*Solanum tuberosum* L. cv. Bintje; Gallou et al. 2010). In this system, the micropropagated plantlets were cultured in an actively growing extraradical mycelium network at an early stage of development. Profuse root colonization was obtained without exceeding the normal duration of the micropropagation period. This system enabled the production of homogenous highly-colonized plantlets in a short time.

AMF are naturally present in the roots of rubber trees in the field (Correa et al. 1993; Schwob et al. 1999; Tawaraya et al. 2003). The inoculation of *H. brasiliensis* seedlings with native AMF resulted in improved plant growth and phosphorus content, with some AMF being more efficient than others (Ikram et al. 1992; Ikram et al. 1993). However, the mycorrhization of *H. brasiliensis* under *in vitro* culture conditions has not been achieved to date. It is obvious that a successful colonization of the plants *in vitro* is a prerequisite towards improved *ex vitro* acclimatization. The objective of the present study was, therefore, to adapt an *in vitro* cultivation system for the colonization of *H. brasiliensis* using the MDP *in vitro* culture system of Voets et al. (2009). The impact of the addition of indole-3-butyric acid (IBA) and 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) to the growth medium, and increase in the atmospheric CO_2 concentration were evaluated on plant root development and root colonization by the AMF.

Materials and Methods

Hevea brasiliensis plantlets

Plantlets of *H. brasiliensis* clone PB 260 (Prang Besar, Malaysia) were produced by somatic embryogenesis (MFP MICHELIN & CIRAD, France). Somatic embryos germinated and plantlets were grown under mixotrophic culture conditions in glass tubes (25 mm diameter x 200 mm height) filled with 30 ml of an agar-gelled culture medium adapted to the embryo germination and growth of *H. brasiliensis* (see Lardet et al. 2007 for details about medium composition). This medium contained 234 mM of sucrose,

but no growth regulators. The plantlets were placed in a growth chamber at 27°C, with a 12 h d^{-1} photoperiod and a photosynthetic photon flux of 60 µmol m^{-2} s^{-1}. Twelve-week-old plantlets with one to three leaves, 4–6 cm stem height, 4–6 cm taproot length, and five to ten early secondary roots (ESR) of 1–3 cm length were considered for the experiments.

Arbuscular mycorrhizal fungus

A strain of *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 was purchased from the Glomeromycota *in vitro* collection (GINCO www.mycorrhiza.be/ginco-bel). The AMF was grown in association with Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 on Petri plates (90 mm diameter x 14 mm height) containing the modified Strullu-Romand (MSR) medium (Declerck et al. 1998) solidified with 3 g l^{-1} Phytagel™, following the method detailed in Cranenbrouck et al. (2005). The Petri plates were incubated in the dark in an inverted position at 27°C for several months until thousands of spores were obtained. Afterwards, the strain was routinely maintained and multiplied in association with *M. truncatula* in a Half-Closed Arbuscular Mycorrhizal–Plant (HAM–P) *in vitro* culture system (Dupré de Boulois et al. 2009). Several thousands of spores were produced in the hyphal compartment within a period of 10 wk.

Mycelium donor plant in vitro culture system

Four-day-old *Medicago truncatula* Gaertn. cv. Jemalong A17 clone seedlings were associated *in vitro* with spores of *R. irregularis* MUCL 41833 to produce a mycelium network (See Voets et al. 2009 for details). Briefly, the MDP *in vitro* culture system consisted of a vented Petri plate (145 mm diameter x 20 mm height) (Greiner bio-one®, Wemmel, Belgium), in which the lid of a 55 mm diameter Petri plate was placed to physically separate a root compartment (RC, i.e., the 55 mm diameter Petri plate) from a hyphal compartment (HC, i.e., the 145 mm diameter Petri plate minus the 55 mm diameter Petri plate; Figure 22). In both compartments, MSR medium without sucrose and vitamins and solidified with 3 g l^{-1} Phytagel™ was added (20 ml in RC and 100 ml in HC).

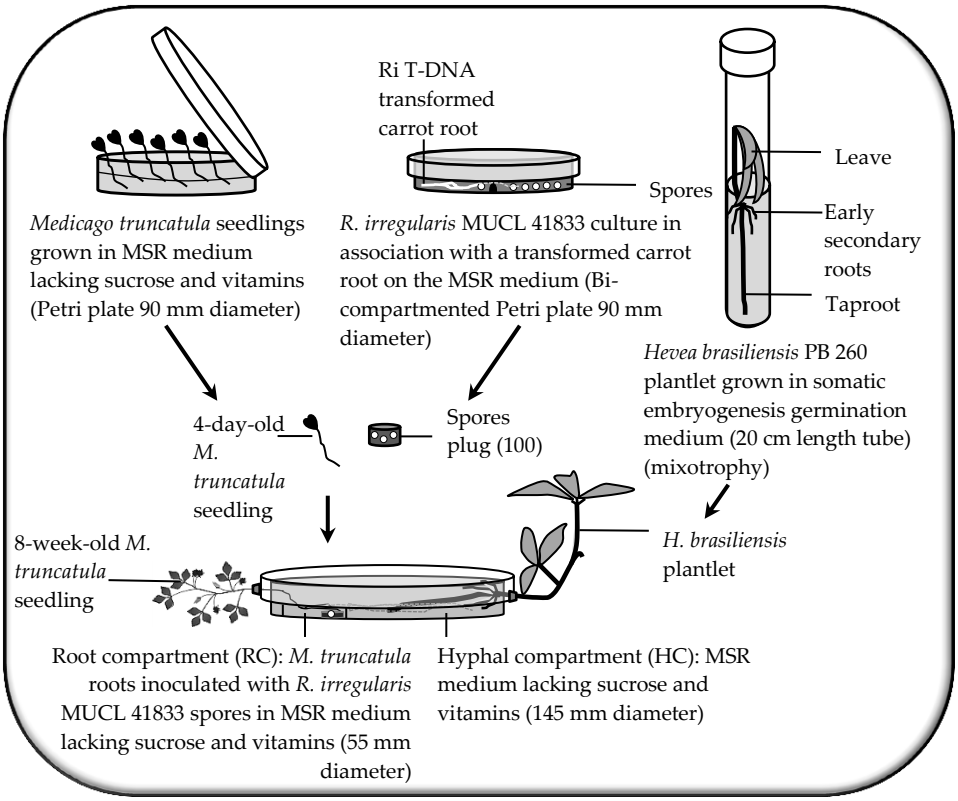

Figure 22. Schematic representation of the procedure used for the *in vitro* mycorrhization of *H. brasiliensis* plantlets.
A mycelium donor plant (MDP) *in vitro* culture system (Voets et al. 2009) with *M. truncatula* associated to AMF in a root compartment (RC) was used to produce a mycelium network in the hyphal compartment (HC) on which somatic-embryogenesis produced *H. brasiliensis* plantlets were placed for colonization (exp. 1).

A four-day-old *M. truncatula* seedling was transferred to the RC, with the roots placed on the surface of the MSR medium and the shoot extending outside both Petri plates via a hole (2–3 mm in diameter) made in the cover and base of the 145 mm diameter Petri plate (Figure 23). A plug of gel containing approximately 100 spores of *R. irregularis* was placed close to the roots.

Another hole (3–4 mm in diameter) was made in the cover and base of the 145 mm diameter Petri plate at the opposite side of the RC (Figure 23). A 12-week-old *H. brasiliensis* plantlet was placed in the system with the roots

plated on the HC and the shoot extending outside the Petri plate via the hole. The system was sealed with ParafilmTM (Pechiney, Plastic Packaging, Chicago, IL, USA), and the holes on both sides of the Petri plate were carefully sealed with silicon grease (VWR International, Belgium) (Figure 22 and 23 A). The MDP *in vitro* culture systems were then placed in a growth chamber under controlled conditions (90% relative humidity, 26°C day 23°C night temperature, 12 h d^{-1} photoperiod under a photosynthetic photon flux of 30 µmol m^{-2} s^{-1} from week one to four and 195 µmol m^{-2} s^{-1} from week five until the end of the experiment.

Experiment 1: Impact of IBA, MES and CO_2 concentration on the* in vitro *colonization of* H. brasiliensis *using the MDP* in vitro *culture system

The experiment was set up using the MDP *in vitro* culture system described previously. Four factors, each represented by two levels, were combined in a factorial design: (i) addition of IBA (pre-treatment of the plantlets vs. non pre-treatment), (ii) AMF (presence versus absence of AMF in the HC), (iii) buffering of the MSR medium (presence or absence of MES in the RC and HC), and (iv) atmospheric CO_2 level (moderate vs. high concentration). In total, 16 treatments were considered with three replicates per treatment (one replicate comprised one *H. brasiliensis* plantlet).

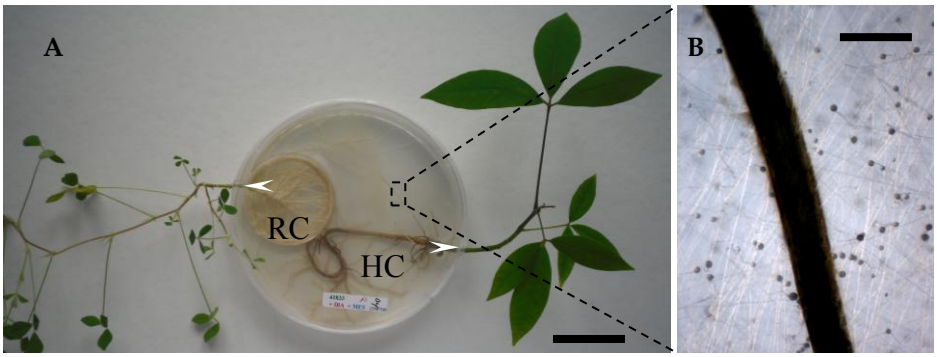

Figure 23. Culture system developed for the *in vitro* mycorrhization of *H. brasiliensis* plantlets.
A. Mycelium Donor Plant (MDP) *in vitro* culture system of *M. truncatula* (left) with a plantlet of *H. brasiliensis* clone PB 260 (right) growing in the mycelium network of *R. irregularis* MUCL 41833. The system allowed the spatial separation of a root compartment (RC) from a hyphal compartment (HC). In the RC, the root system of *M. truncatula* was associated to the AMF. After 7 weeks, the mycelium of the AMF extended from the RC to the HC and the roots of the *H. brasiliensis* plantlet were placed in contact with this extraradical mycelium. The shoots of *M. truncatula* and *H. brasiliensis* extended outside the MDP *in vitro* culture system via a hole (white arrows), bar = 42 mm. **B.** Hyphae and spores of *R. irregularis* around a *H. brasiliensis* root, bar = 200 μm.

For the IBA treatment, the *H. brasiliensis* plantlets were exposed (+IBA) or not (-IBA) to 10 μM IBA potassium salt (Fluka BioUltra Sigma Aldrich®, Bornem, Belgium) added into the culture medium for 1 week, before the transfer to the MDP *in vitro* culture systems. For the AMF treatment, the plantlets developed in the presence (+AMF) or absence (-AMF) of the mycelium network in the HC as described previously. For the MES treatment, the MSR medium was supplemented (+MES) or not (-MES) with 10 mM of MES (Fluka BioUltra Sigma Aldrich®, Bornem, Belgium) solution to stabilize the pH at 5.5. For the CO_2 treatment, the plantlets were maintained under high (approximately 1000 μmol mol^{-1}) atmospheric CO_2 concentration (+CO_2), or under a moderate (approximately 500 μmol mol^{-1}) atmospheric CO_2 concentration (-CO_2).

Starting from week three, MSR medium without sucrose and vitamins and solidified with Phytagel™, was added every 2 weeks in the RC and HC of

the systems (10 ml in RC and 30 ml in HC) to maintain the volume of the medium and the nutrient content in the compartments. For the plantlets treated with MES, the MSR medium was supplemented with this buffer. After 7 weeks of incubation, the AMF started to cross the partition wall separating the RC from the HC and developed profusely in the HC forming a dense extraradical mycelium network (Figure 23 B).

Twelve weeks after the start of the experiment (i.e., after the transfer of the plantlets into the MDP *in vitro* culture systems), shoot and root length and root colonization were measured. Shoot length was measured from the base of the shoot (junction with the lateral roots) to the base of the petiole of the oldest leaf. Root length was estimated using the modified line intersect method (Newman 1966). For AMF colonization, roots were cleared in 10% KOH overnight at room temperature, followed by 4 h at 50°C. The roots were then rinsed with distilled water, bleached with 3% H_2O_2 at 50°C for 5 min, and stained with an ink-vinegar solution (5% *v*/*v* vinegar with 5% *v*/*v* Parker® blue ink; Vierheilig et al. 1998) at 50°C for 5 min. The entire root system was subsequently cut into 2 cm pieces and mounted on microscope slides. The root pieces were examined for AMF colonization under a compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) at x125 magnification. The percentage of total root colonization and percentages of arbuscules and intraradical spores/vesicles were estimated using the magnified intersects method (McGonigle et al. 1990). For each plant, a minimum of 150 root intersections were evaluated.

Experiment 2: Dynamics of* H. brasiliensis *root colonization under elevated and moderated CO_2 conditions using the MDP* in vitro *culture system supplemented with MES

Twelve-week-old *H. brasiliensis* plantlets were placed in the MDP *in vitro* culture systems as described previously. However, in this experiment, the plantlets were transferred into the HC 7 weeks after association of the AMF to *M. truncatula* in the RC, i.e. at the time when the mycelium crossed the partition wall separating the RC from the HC. At this time, considered as the start of the experiment, one new opening (3–4 mm in diameter) was made in the HC and one *H. brasiliensis* plantlet was placed with the roots in contact with the mycelium network. The Petri plates were sealed as described previously. As in experiment one, starting from week three, MSR medium without sucrose and vitamins and amended with 10 mM MES was added every 2 weeks in both compartments (10 ml in the RC and 30 ml in the HC). The experimental conditions where the same as in experiment one with half

of the plantlets transferred in a growth chamber with approximately 1000 μmol $mol^{-1}$of CO_2 and the other half in a growth chamber with approximately 500 μmol mol^{-1} CO_2. Destructive measures of shoot and root lengths and root colonization were made 9, 11, and 13 weeks after the transfer of the *Hevea* plantlets into the MDP *in vitro* culture systems. Four plantlets (replicates) were sampled at each time. The root systems were collected, stained, and quantified for root colonization as described previously.

Statistical analyses

Data analysis was performed with the statistical software Statistica® for Windows (StatSoft 2001). For the percentage of total, arbuscules and intraradical spores/vesicles root colonization, the values were arcsine √(x/100) transformed before statistical analysis. After verification of the normal distribution, homogeneity of the variances and residuals' independency of the data, two or three-way ANOVA ($P \leq 0.05$) was applied. The Tukey honest significant difference test was used to identify the significant differences ($P \leq 0.05$).

Results

Experiment 1: Impact of IBA, MES and CO_2 concentration on the* in vitro *colonization of* H. brasiliensis *using the MDP* in vitro *culture system

At the start of the experiment, the root system of the *H. brasiliensis* plantlets consisted of a taproot and five to ten lateral roots anchored at the base of the shoot (usual traits for *Hevea* plantlets). Regardless of the treatment, the length of the taproot increased until the end of the experiment (week 12). New lateral roots were observed emerging from the taproots, 7 to 8 weeks after the transfer of the plantlets into the culture systems, and at the base of the shoot starting from week six. Most of the initial lateral roots did not grow and no new ramifications were formed on these roots. These primary lateral roots have been described as transitory roots by Leroux and Pagès (1994).

Twelve weeks after the transfer in the MDP *in vitro* culture systems (i.e., 5 weeks after contact with the extraradical mycelium network), all the plantlets were harvested and measured. No root colonization was observed for the plantlets pre-treated with IBA, alone or in combination with MES or CO_2. Therefore, these plantlets were discarded from further analyses. Root colonization was observed for the *Hevea* plantlets grown in the HC plus or minus MES and grown under 500 as well as 1000 μmol mol^{-1} CO_2 (Table 3).

A typical Arum-type colonization was observed, with intraradical hyphae growing along the intercellular spaces. Groups of arbuscules and intraradical spores/vesicles were formed densely in some root fragments. The total root colonization remained low with values ranging from 2.3% to 9.2% (Table 3). The CO_2 concentration alone had no significant effect on root colonization. MES significantly increased total root colonization as well as the percentage of arbuscules and intraradical spores/vesicles. The plantlets grown under elevated CO_2 concentration and MES added to the MSR medium had a significantly higher total root colonization (post-hoc analysis), while no effect was observed on the percentages of arbuscules and intraradical spores/vesicles (Table 3).

With the exception of the combination AMF x MES, no significant effect of CO_2, MES, and AMF or combinations of these factors was noted on the shoot length of the plantlets (Table 4). Post-hoc analyses showed that plantlets in the treatments +AMF x +MES and –AMF x –MES had significantly smaller shoots than plantlets in the treatment –AMF x + MES. Root length was significantly affected by the interaction AMF x CO_2 x MES (Table 4). Post-hoc analyses showed that at a high CO_2 level, MES and AMF treatment did not significantly influence the root length, but at the lower CO_2 level, root length was higher for treatments –AMF x +MES and +AMF x –MES than for the other combinations.

Table 3. Impact of 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) and CO_2 concentration on the *in vitro* colonization of *H. brasiliensis* clone PB 260

Treatment		**Root Colonization*(%)**		
CO_2 level (μmol mol^{-1})	MES (mM)	Total	Arbuscules	intraradical spores/vesicles
1000	10	9.2 ± 0.3a	5.6 ± 0.8a	5.8 ± 1.0a
	0	2.3 ± 0.6b	1.4 ± 0.4a	1.3 ± 0.5a
500	10	4.8 ± 0.4b	3.7 ± 0.9a	3.9 ± 0.1a
	0	4.1 ± 1.3b	3.0 ± 1.2a	2.6 ± 1.1a
Significance of treatment				
CO_2		NS	NS	NS
MES		<0.05	<0.05	<0.05
CO_2 x MES		<0.05	NS	NS

Data represent means ±SE of three replicates (two-way ANOVA, $P \leq 0.05$). For each *column*, values followed by a different *letter* differ significantly following post-hoc analyses of the significant interaction CO_2 x MES (Tukey's HSD test, $P \leq 0.05$).

*Root colonization was quantified after 12 weeks of growth *in vitro*, corresponding to 5 weeks of contact with the extraradical mycelium of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis* MUCL 41833

Table 4. Impact of arbuscular mycorrhizal fungi (AMF), 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES), and CO_2 concentration on the plant growth of *H. brasiliensis* PB 260 grown in the mycelium donor plant (MDP) *in vitro* culture system

AMF	CO_2 level (µmol mol^{-1})	MES (mM)	Shoot length*(cm)	Root length*(cm)
Absent	500	0	6.3 ± 1.1a	66.5 ± 9.8a
		10	10.2 ± 2.5a	112.1 ± 20.6bc
	1000	0	7.7 ± 0.8a	67.9 ± 13.4a
		10	8.1 ± 0.6a	66.6 ± 6.1a
Present	500	0	8.6 ± 0.8a	113.9 ± 15.2c
		10	7.3 ± 1.8a	52.6 ± 16.0a
	1000	0	8.2 ± 1.8a	74.1 ± 10.9ab
		10	6.6 ± 2.0a	63.4 ± 12.3a
Significance of treatment				
AMF			NS	NS
CO_2			NS	<0.01
MES			NS	NS
AMF x CO_2			NS	NS
AMF x MES			<0.05	<0.0001
CO_2 x MES			NS	NS
AMF x CO_2 x MES			NS	<0.001

Data represent means ±SE of three replicates (three-way ANOVA, $P \leq 0.05$). For each *column*, values followed by a different *letter* differ significantly following post-hoc analyses of the significant interaction AMF x CO_2 x MES. Note that post-hoc analyses of lower order interactions were not shown (Tukey's HSD test, $P \leq 0.05$). *Data were recorded after 12 weeks of growth *in vitro*, corresponding to 5 weeks of contact with the extraradical mycelium of the AMF *Rhizophagus irregularis* MUCL 41833.

Experiment 2: Dynamics of* H. brasiliensis *root colonization under elevated and moderated CO_2 conditions using the MDP* in vitro *culture system supplemented with MES

The length of shoots and roots of the plantlets significantly increased over time (Table 5). The shoot and root lengths were significantly higher under elevated CO_2 as compared to the moderate CO_2 concentration. A significant interaction between Time x CO_2 was noticed on root length, but not on shoot length.

Seven weeks after placement of the *Hevea* plantlets into the mycelium network, several hyphopodia were observed on the surface of the early secondary roots emerging from the taproot (secondary roots of the acropetal sequence) and some intraradical spores/vesicles were found in the older parts of some lateral roots (data not shown). At week nine, intraradical spores/vesicles and arbuscules were observed in the plantlets grown under both CO_2 treatments. The total root colonization and percentages of arbuscules and intraradical spores/vesicles significantly increased with time regardless of the concentration of CO_2 (Table 5). The concentration of carbon dioxide affected these parameters with higher colonization levels under 1000 μmol mol^{-1} CO_2. The root colonization was positively correlated (linear regression estimated at α=0.05) with the length of the roots under high (+CO_2: P=0.002; R^2=88.6%) as well as low (P=0.005; R^2=82.1%) CO_2 concentrations.

Table 5. Effect of two levels of CO_2 on the arbuscular mycorrhizal fungi (AMF) colonization, root and shoot lengths of *H. brasiliensis* clone PB 260 plantlets after 9, 11, and 13 weeks of growth in contact with the extraradical mycelium of the AMF *Rhizophagus irregularis* MUCL 41833

Level of CO_2 (µmol mol^{-1})	**Time (weeks)**	**Root length (cm)**	**Shoot length (cm)**	**Total root colonization (%)**	**Arbuscules (%)**	**intraradical spores/ vesicles (%)**
500	9	18 ± 5a	6.9 ± 1.5a	1.8 ± 0.5a	1.4 ± 0.5a	1.1 ± 0.5a
	11	41 ± 9bc	8.8 ± 0.6a	6.3 ± 1.3a	5 ± 1.3a	4.2 ± 1.0a
	13	50 ± 6c	9.8 ± 1.7a	16.7 ± 2.6a	15.1 ± 2.4a	13.6 ± 2.2a
1000	9	26 ± 6ab	8.8 ± 1.7a	2 ± 0.4a	1.7 ± 0.4a	1.5 ± 0.3a
	11	70 ± 7d	9.1 ± 0.8a	10 ± 1.5a	8.7 ± 1.8a	7 ± 1.5a
	13	75 ± 2d	11.6 ± 2.2a	23 ± 2.3a	21.5 ± 2.6a	19.1 ± 2.6a
Significance of the treatment						
CO_2		<0.001	<0.001	<0.05	<0.05	<0.05
Time		<0.001	<0.05	<0.001	<0.001	<0.001
CO_2 x Time		<0.05	NS	NS	NS	NS

Data represent means ±SE of four replicates (two-way ANOVA, $P \leq 0.05$). For each *column*, values followed by a different *letter* differ significantly following post-hoc analyses of the significant interaction CO_2 x Time (Tukey's HSD test, $P \leq 0.05$)

Discussion

In this study, we report the successful *in vitro* mycorrhization of *Hevea brasiliensis*. Root colonization was established via actively growing hyphae extending from an extraradical mycelium network using the autotrophic MDP *in vitro* culture system developed by Voets et al. (2009). Under an elevated CO_2 concentration and addition of MES in the growth medium, the total root colonization reached 23 ± 2.3% after 13 weeks of growth into the mycelium network, with numerous arbuscules (21.5 ± 2.6%) and vesicles (19.1 ± 2.6%) produced.

Regardless of the treatment, five types of roots, similar to the roots described from seedlings grown under greenhouse conditions (Le Roux and Pagès 1994; Le Roux and Pagès 1996), were observed: (1) the taproot, (2) the early secondary roots (ESR), (3) the secondary roots of the acropetal sequence (SRAS), (4) the late secondary roots (LSR), and (5) the tertiary roots. In the taproot, colonization was restricted to the youngest segment (~3 mm diameter) that developed after plant transfer to the MDP *in vitro* culture systems, while in the older parts no colonization was observed. The colonization of the existing roots (i.e., the roots formed during the mixotrophic phase, such as the secondary roots) was erratic with only some intraradical spores/vesicles occasionally found in the older tissues next to the newly formed ones. To the contrary, the presence of arbuscules and intraradical spores/vesicles was observed within the root tissues that were newly formed. Late secondary roots appeared from the sixth week of culture in the MDP *in vitro* culture system, but their colonization was low. This could be explained by the tendency of these roots to grow outside the medium. Most of these roots did not grow into the MSR medium, and therefore were not in contact with the mycelium network. Secondary roots of the acropetal sequence and tertiary roots were formed from the fifth and the ninth week of growth under autotrophic culture conditions, respectively. Most of these roots were colonized and contained arbuscules and intraradical spores/vesicles.

IBA, MES, and CO_2 were assayed to stimulate root growth and development and thus favoring root colonization by the AMF. IBA has been reported to improve *in vitro* rooting of different plants (Díaz-Pérez et al. 1995; Fabbri et al. 2004; Padilla et al. 2006). For example, the incubation in 10 μM of IBA solution for 1 week increased the number of fine roots produced in maize (Kaldorfa and Ludwig-Müller 2000). However, under our experimental conditions, a similar exposure to IBA did not stimulate the formation of new roots or impact the root morphology or length (data not shown). This could

be related to the sensitivity of the root tissues to the IBA treatment, and/or the micropropagation technique. For instance, de Klerk et al. (1999) applied pulses of 2.5 μM IBA at different phases of the rooting process of apple microcuttings. They observed rooting stimulation only when IBA was applied the first 72 h, but after 96 h, the auxin was no longer required and even became inhibitory. For *H. brasiliensis*, it may be possible to obtain a positive response on root generation after IBA application using plantlets produced by microcuttings instead of somatic embryogenesis, or changing the concentration/timing of the IBA treatment.

No root colonization was observed in plantlets pre-treated with IBA. This may suggest an indirect effect of this plant growth regulator on the AMF via the plant or a direct effect via the release of IBA in the MSR medium, since the roots were not completely cleaned-free from the germination medium when transplanted in the extraradical mycelium network growing in the HC. IBA was reported to inhibit the proliferation of hyphae of *G. fistulosum* and *G. mosseae* (Gryndler et al. 1998) suggesting that this auxin may potentially impact root colonization.

On the contrary, the MES buffer positively influenced root colonization. The role of MES on AMF development *in vitro* has been observed in root organ cultures (ROC – Douds Jr 1997; Pawlowska et al. 1999; Karandashov et al. 2000) and in the autotrophic *in vitro* culture of AMF associated to banana plantlets (Koffi et al. 2009). Stabilization of the medium pH appeared as a possible mechanism, but the actual origin of this influence on the AMF development remains unclear. In this work, root colonization was increased by addition of MES in the MSR medium, and the combination with CO_2 also resulted in higher root colonization of the plantlets. The addition of this buffer did not affect the root length of the *H. brasiliensis* plantlets, although the stimulation of the *in vitro* rooting of other plants has been reported (Harbage and Stimart 1996).

High concentrations of CO_2 have been used to improve the growth of several plants (Pospísilová et al. 2007). The augmentation of the root growth (root number, length, and biomass) in woody plants cultured in rooms with enriched CO_2 conditions (1000-1,200 μmol mol^{-1}) during the *in vitro* acclimatization in autotrophic conditions has been observed for different species (Kozai and Kubota 2001; Mosaleeyanon et al. 2004). In our study, the positive effect of 1000 μmol mol^{-1} CO_2 treatment was clearly observed in the mycorrhized plantlets (second experiment). Root length significantly increased over time for colonized *H. brasiliensis* plantlets exposed to high level of CO_2 treatment in combination with MES. This combination also

resulted in a significant increase of root colonization. The stimulatory effect of CO_2 on the AMF growth (hyphal length elongation) was earlier demonstrated in ROC of *Gigaspora margarita* (Becard and Piché 1989) applied singly or in association with flavonoids (Becard et al. 1992). The enhancement of the mycorrhization of strawberry (Elmeskaoui et al. 1995) and potato exposed to high CO_2 concentration *in vitro* was also reported (Louche-Tessandier et al. 1999).

The correlation found between the time course of root colonization and the root length, and the presence of intraradical spores/vesicles and arbuscules mostly limited to younger roots, suggest that the establishment of root colonization was strongly dependent on the emergence of new roots in the autotrophic culture of the plantlets, which did not seem to be influenced by the CO_2 level. This corroborated earlier findings with *Plantago lanceolata* and *Trifolium repens* (Staddon et al. 1998). Indeed, the symbiosis with AMF has shown mixed responses to CO_2 enrichment (Staddon and Fitter 1998; Fitter et al. 2000; Treseder and Allen 2000). Positive stimulation, no change or even decreases in the percentage of root length colonized or hyphal biomass has been described (Staddon and Fitter, 1998).

It is important to note that in the second experiment, the *H. brasiliensis* plantlets were in contact with the mycelium network during the 13 weeks period (versus 5 weeks in the first experiment). Nevertheless, this early contact and longer exposure did not accelerate the onset of the root colonization that was almost exclusively restricted to the newly produced roots with only few traces in those produced during the mixotrophic phase before transfer in the MDP *in vitro* culture systems.

This study is the first to report on the *in vitro* mycorrhization of *H. brasiliensis* plantlets. We validated the use of the MDP *in vitro* culture system as a suitable method to produce a mycelium network able to actively colonize the rubber tree plantlets. Typical intraradical structures (hyphae, arbuscules, and intraradical spores/vesicles) were observed in the roots. It appeared that the most adequate conditions for root colonization were elevated CO_2 (1000 $\mu mol\ mol^{-1}$) content in the atmosphere of the growth chamber and 10 mM buffer MES addition to the MSR culture medium. Root colonization was obtained after several weeks of *in vitro* culture, but was mostly restricted to the new roots, suggesting that actively growing roots are mandatory for a strong colonization. This study suggests the necessity to evaluate additional *in vitro* autotrophic culture conditions that could stimulate the early production of roots in order to reduce the time necessary to obtain mycorrhized plantlets. It also opens the door to test *in vitro* produced

mycorrhized *Hevea* plantlets under greenhouse or nursery conditions, to evaluate whether this method could improve further development during the *ex vitro* acclimatization.

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Chapter 3

Effect of activated charcoal and pruning of the taproot on the *in vitro* mycorrhization of *Hevea brasiliensis* Müll. Arg.

Preface

The application of elevated concentration of CO_2 in the growth chamber and the addition of MES buffer to the culture medium stimulated the *in vitro* colonization of the *H. brasiliensis* plantlets (**Chapter 2**). However, the root production was not significantly enhanced by these treatments. In addition, a long period (approx. 9 weeks) remained necessary for root colonization and production of arbuscules. Here, new parameters were investigated to stimulate root production and colonization. Activated charcoal (AC) was selected because it has been reported to favor the growth and the root production of various plant species cultured *in vitro* (Thomas 2008). Pruning of the taproot was also assayed to induce root production, although it involved a strong modification of the plant root system morphology. We evaluated the effect of each treatment and their combination on root growth and colonization and on the enzymatic activity of the extraradical mycelium network of the AMF *R. irregularis*.

Effect of activated charcoal and pruning of the taproot on the *in vitro* mycorrhization of *Hevea brasiliensis* Müll. Arg.

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Abstract

In vitro mycorrhization of *Hevea brasiliensis* under autotrophic culture conditions is a promising methodology to produce plantlets better adapted to overcome stresses during acclimatization. To achieve this objective, optimization of root production and subsequent colonization is necessary. Plantlets of *H. brasiliensis* clone PB 260 were grown in contact with the extraradical mycelium network of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* MUCL 41833. Two factors, i.e. the addition of activated charcoal (AC) to the growth medium and the pruning of the taproot were evaluated on root growth and colonization. None of the treatments stimulated the early formation of new roots. However, total root length, total root colonization, and production of arbuscules and intraradical spores/vesicles were significantly higher in plantlets grown in the presence of AC (especially after 13 weeks of culture). To the contrary, total root colonization was significantly lower in the pruned plantlets, while total root length and arbuscule formation were not impacted. None of the treatments affected the enzymatic activities (succinate dehydrogenase and alkaline phosphatase) measured on the extraradical mycelium of the AMF. From these results, it appeared that the addition of AC to the culture medium favored root growth and mycorrhization of rubber plantlets under *in vitro* culture conditions, while taproot pruning did not affect these parameters.

Key Words activated charcoal • taproot pruning • natural rubber • arbuscular mycorrhizal fungi • extraradical mycelium network • autotrophy • *in vitro* culture

Introduction

Hevea brasiliensis Müll. Arg. is a tree crop cultivated worldwide for the production of natural rubber. This raw material is used in the manufacture of numerous rubber goods, especially tires, and its demand is increasing constantly (Nair 2010). To satisfy the market, the development and propagation of genotypes with enhanced latex production has become a priority for the natural rubber industry.

Several vegetative multiplication techniques are currently applied in nursery and under *in vitro* culture conditions (Perrin et al. 1994; Carron et al. 1995; Lardet et al. 2007; Carron et al. 2009; Vaysse et al. 2009; Montoro et al. 2012). In particular, *in vitro* micropropagation techniques such as somatic embryogenesis or microcuttings allows for the mass propagation of selected clones that will be later acclimatized in the nursery. Rapid growth recovery and high vigor are necessary before final transplantation to the field (Carron et al. 2009; Montoro et al. 2012).

Different *in vitro* and/or *ex vitro* treatments have been tested to improve growth and physiology of *in vitro* produced plantlets before acclimatization and transplantation into the field. For instance, the modification of the culture conditions, the management of the *ex vitro* acclimatization environment (Chandra et al. 2010) and the inoculation with beneficial soil microorganisms (Nowak 1998; Hazarika 2006; Liu and Yang 2008) are options currently considered.

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with nearly 80% of vascular plants. These obligate root symbionts have been reported to improve growth and resistance to biotic and abiotic stresses of numerous crops (Smith and Read 2008). AMF can be mass-produced in controlled conditions (e.g. greenhouse, *in vitro* root organ culture; see IJdo et al. 2011 for a review), allowing their utilization for the sustainable management of agro-ecosystems (Gianinazzi et al. 2010). In addition, their application during the acclimatization phase of micropropagated plantlets has been reported to improve plant performance at transplantation to the field (Kapoor et al. 2008).

Recently, Sosa-Rodriguez et al. (2013a) achieved the association of *H. brasiliensis* plantlets with the AMF *Rhizophagus irregularis* MUCL 41833 under *in vitro* autotrophic culture conditions. The highest root colonization was obtained under CO_2 enriched atmosphere (1000 μmol mol^{-1}) combined to the addition of 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) buffer (10 mM) to the growth medium. Nevertheless, the colonization only

established after a long period (7 weeks for the proliferation of intraradical hyphae and at least 9 weeks for the production of the first arbuscules), and appeared to be restricted almost exclusively to the newly produced roots. Recent findings suggested that the delay in the colonization of *H. brasiliensis* roots may be related to the exudation of inhibitory molecules by the plant roots (Sosa-Rodriguez et al. 2013b). Indeed, the presence of inhibitory compounds that interfere with the plant root colonization by AMF has been reported previously (see Vierheilig and Bago 2005 for a review).

According to several authors (Hepper 1981; Hepper 1985; Afek et al. 1990; Amijee et al. 1993; Brundrett 2002), root colonization by AMF is closely related with root anatomy and age. For instance, Guo et al. (2008) observed that lateral young roots are more susceptible to AMF colonization than older roots with secondary development. Besides, young sections of growing roots appeared more susceptible to AMF penetration, especially in areas where exodermis is developing (i.e. before complete suberization of the exodermal root cells; Vidal et al. 1992), and/or in zones with presence of specific passage cells (Brundrett 2002; Sharda and Koide 2008).

H. brasiliensis young plants have hierarchical root morphology, presenting a strong taproot and several lateral roots. The taproot has a large apical diameter, well-developed stele and primary and secondary growth. Lateral roots do not have secondary growth. Interestingly, each type of roots has a specific patron of genes expression that suggests the specialization of the root system to accomplish different functions (Putranto et al. 2012).

Indeed, the root system of an adult rubber tree keeps the root hierarchy, with the taproot which gives stability and anchorage to the plant, playing a role in the longitudinal conduction of water and nutrients, and a superficial network of lateral roots, mostly involved in nutrient uptake, which have a short lifespan, and are capable of rapid regeneration, thus being involved in fast responses to environmental signals (Delabarre and Serier 1995; Carron et al. 2000; Putranto et al. 2012).

Altering the morphology and growth of the root system of H. brasiliensis plantlets may enhance the production of secondary roots (Thaler and Pagès 1997). These authors demonstrated that taproot pruning induced a rapid regeneration of the excised root, and the growth of numerous secondary roots from the neo-formed taproot. Therefore, such treatment may possibly induce the early and abundant production of secondary or lower order roots, potentially more susceptible to colonization by AMF.

In vitro plant growth and root production can also be stimulated by the addition of specific compounds, such as activated charcoal (AC), to the culture medium (Thomas 2008; de Klerk et al. 1999; de Klerk 2002). Activated charcoal is an organic black carbon material frequently added to promote rooting and to adsorb exogenous or endogenous substances (e.g. inhibitory compounds such as the polyphenols and their oxides, ethylene, and/or other plant hormones), that may accumulate in the culture medium and impact the normal plant growth (Pan and Staden 1998). The stimulation of rooting in micro-cuttings (Carron *personal communication*) and the embryo development (Etienne et al. 1993; Blanc et al. 1999) of *H. brasiliensis* have been observed in presence of AC.

The adsorptive capacity of AC was also exploited in allelopathy studies. For instance, Inderjit and Callaway (2003) demonstrated that the negative effects of allelopathic plants on the growth of associated plant species can be alleviated when inhibitory compounds exuded by the roots are sequestered by AC added to the growth substrate.

Interestingly, AC has been reported to stimulate the development or mycorrhizal fungi. Kottke et al. (1987) observed *in vitro* that the association of different coniferous tree seedlings with ectomycorrhizal (ECM) fungi was only achieved in presence of AC. The positive influence of biochar (a charcoal variant used as soil amendment) and AC on AMF soil diversity and on root colonization has been reported several times (Daniels and Trappe 1980; Warnock et al. 2007; Rillig et al. 2010; Solaiman et al. 2010; Elmer and Pignatello 2011). Together, these results suggested that AC could also have beneficial effects on the development of the AM symbiosis under *in vitro* culture conditions.

In the present study, we used a mycelium donor plant (MDP) *in vitro* culture system for the mycorrhization of *H. brasiliensis* (Sosa-Rodriguez et al. 2013a). In order to fasten lateral root growth, and to improve the *in vitro* colonization of the rubber plantlets, we evaluated the impact of taproot pruning and addition of AC to the growth medium on root production of *H. brasiliensis* clone PB 260 and subsequent colonization by *Rhizophagus irregularis* MUCL 41833, and on the enzymatic activity of the AMF extraradical mycelium.

Materials and methods

Hevea brasiliensis

Plantlets of *H. brasiliensis* clone PB 260 were produced *in vitro* by somatic embryogenesis (MFP MICHELIN & CIRAD, France). The plantlets were maintained in glass tubes filled with a culture medium specifically adapted to the embryo germination and *in vitro* propagation of *H. brasiliensis* (see Lardet et al. 2007 for details). The tubes were placed in a growth chamber at 27 °C, with a 12 hd^{-1} photoperiod and a photosynthetic photon flux of 60 $\mu mol\ m^{-2}\ s^{-1}$ until transfer of the plantlets to the Mycelium donor plant (MDP) *in vitro* culture systems (see below). Twelve-week-old plantlets with one to three leaves, 4-6 cm stem height, 4-6 cm taproot length and five to ten early secondary roots (ESR) of 1-3 cm length were used in the experiments.

Arbuscular mycorrhizal fungus

A root organ culture (ROC) of *Rhizophagus irregularis* (Błaszk, Wubet, Renker & Buscot) C. Walker & Schuessler (2010) [as 'irregulare'] MUCL 41833 (Synonymy: *Glomus irregulare*) was purchased from the Glomeromycota *in vitro* collection (GINCO www.mycorrhiza.be/ginco-bel). The strain was initially maintained in ROC (Cranenbrouck et al. 2005). Afterwards, it was mass-produced in association with *Medicago truncatula* Gaertn. cv. Jemalong A17 (SARDI, Adelaide, Australia) in a Half-Closed Arbuscular Mycorrhizal–Plant (HAM–P) *in vitro* culture system (Voets et al. 2005). Several thousands of spores were produced in the hyphal compartment (HC) of this system within a period of 10 weeks.

Experimental systems

The Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) for the *in vitro* mycorrhization of *H. brasiliensis* plantlets (Sosa-Rodriguez et al. 2013a) was used. The system consisted of two compartments (i.e. a 55 mm diameter Petri plate placed inside a 145 mm diameter Petri plate), allowing the physical separation of the plant roots associated to the AMF in the root compartment (RC), from the extraradical mycelium that spread in the hyphal compartment (HC; Fig. 24 A). In both compartments, modified Strullu-Romand (MSR) medium (Declerck et al. 1998) without sucrose and vitamins, solidified with 3 $g\ l^{-1}$ Phytagel™ (Sigma-Aldrich, St. Louis, USA) and amended with 10 mM MES buffer pH 5.5 (Fluka BioUltra Sigma Aldrich®, Bornem, Belgium), was added (20 ml in RC and 100 ml in HC). In order to facilitate the addition of MSR medium during the time-course of the experiment, the top of a 50 ml centrifuge tube with

screw cap (30 mm diameter x 35 mm length) was glued to the lid of the system (Fig. 24 B). The lid was gamma sterilized (25 KGy) before utilization.

One *M. truncatula* seedling was placed in the RC of each system. A plug of gel of 0.4 cm^3 containing approximately 100 spores of *R. irregularis* was placed close to the roots. The systems were sealed and incubated in a growth chamber under controlled conditions (22°C/18°C day/night temperature, 70% relative humidity, 16 hd^{-1} photoperiod and photosynthetic photon flux of 225 μmol m^{-2} s^{-1}). Starting from week 3, the systems were supplied weekly with 0.5 ml of liquid MSR medium (concentrated 10 times), added to a hole of 1.75 cm^3 made in the gelled medium of the RC with a cork borer (Fig. 24 A; Dupré de Boulois et al. 2006). The MSR medium was added via the tube. Seven weeks after association of the AMF to *M. truncatula*, the extraradical mycelium (ERM) of *R. irregularis* crossed the partition wall separating the RC from the HC and started to spread in the HC. At that time, one twelve-week-old *H. brasiliensis* plantlet was transferred into the HC of each system, with the roots in contact with the mycelium network and the shoot extending outside the system (Fig. 24). This transfer was considered as the start of the experiment. From this moment, 0.5 ml of the liquid MSR medium described above was added weekly via the tube, in a hole of 1.75 cm^3 made in the HC (Fig. 24 A and B).

All the systems were placed in a growth chamber under CO_2 enriched atmosphere (1000 μmol mol^{-1}; see Sosa-Rodriguez et al. 2013a for details), at 26°C/23°C day/night temperature, 70% relative humidity and 12 hd^{-1} photoperiod and a photosynthetic photon flux of 60 μmol m^{-2} s^{-1} from week one to four and 195 μmol m^{-2} s^{-1} from week five until the end of the experiment.

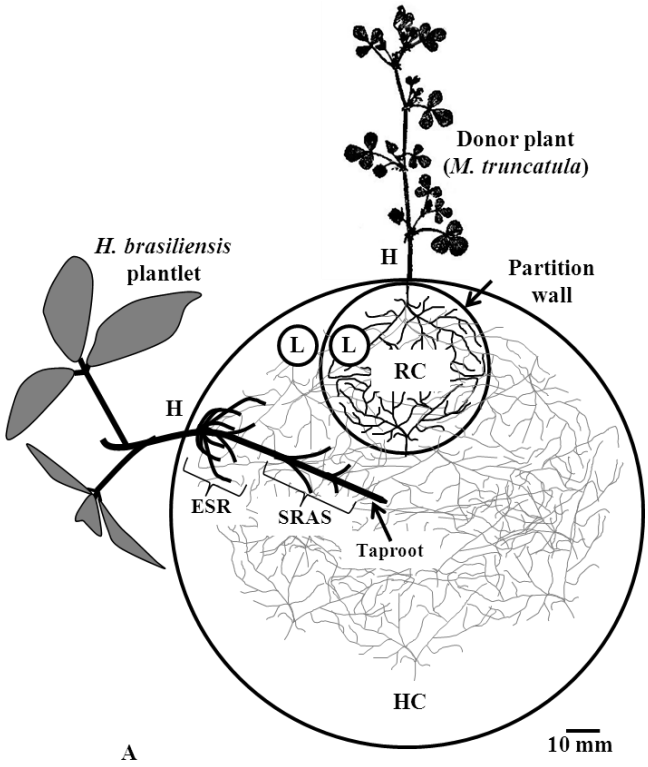
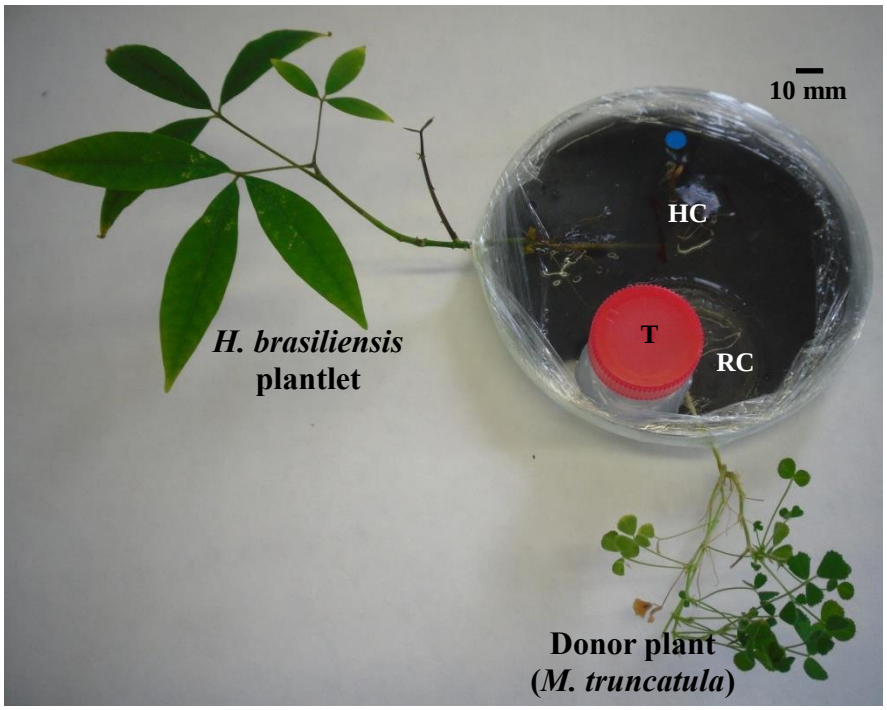


Figure 24. **A.** Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) to study the impact of activated charcoal (AC) and pruning (P) of the taproot on *Hevea brasiliensis* plantlets.
Schematic representation of the system with a seedling of *M. truncatula* developing in a root compartment (RC) in association with the AMF *Rhizophagus irregularis*. The AMF crossed the partition wall and developed in the hyphal compartment (HC) in contact with the rubber plantlet root system (early secondary roots -ESR, secondary roots of the acropetal sequence -SRAS and taproot). The shoot of the plants developed outside the system via a hole (H). Modified Strullu-Romand (MSR) liquid medium was added regularly to the RC and HC in 1.75 cm^3 holes (L) made in the solid MSR medium (see details in text).



B

Figure 24. **B.** Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) to study the impact of activated charcoal (AC) and pruning (P) of the taproot on *Hevea brasiliensis* plantlets.
Culture system with activated charcoal (AC) added to the medium, showing the position of the tube with screw cap (T) on the system lid, the *M. truncatula* donor plant, and the *H. brasiliensis* plantlet.

Treatments

Two factors, the addition of activated charcoal (AC) and the pruning (P) of the taproot, each represented by two levels, were combined in a factorial design. For the treatment with activated charcoal (+AC), the MSR medium in both compartments was supplemented with 1 g l^{-1} of AC (Sigma-Aldrich, St. Louis, USA), added during the medium preparation (i.e. before autoclaving). A control without AC added to the MSR medium (-AC) was also included. For the taproot pruning (+P) treatment, the taproot of the *H. brasiliensis* plantlet was pruned 3 cm below the early secondary roots (ESR), or kept intact (-P). In total, four treatments were considered (+AC-P; +AC+P; -AC-P; -AC+P). The plantlets were harvested after 7, 9, 11, 13 or 15 weeks of

growth in contact with the ERM network of *Rhizophagus irregularis* MUCL 41833. Four replicates were considered per treatment and harvest time.

Root colonization

Root colonization was estimated after 7, 9, 11, 13 or 15 weeks of contact of *H. brasiliensis* with the ERM of *R. irregularis*. The entire root system of each plantlet was collected. Roots were cleared in 10% KOH at 80°C for 4 hours, rinsed with distilled water, bleached with 3% H_2O_2 at 80°C for 3 minutes, and stained with an ink-vinegar solution (5% v:v vinegar with 5% v:v Parker® blue ink; Vierheilig et al. 1998) at 80°C for 5 minutes. Roots were subsequently cut in 2 cm length pieces and mounted on microscope slides. The root pieces were examined for AMF colonization under a compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) at x125 magnification. The percentage of total root colonization and percentages of arbuscules and intraradical spores/vesicles were estimated using the magnified intersects method (McGonigle et al. 1990). For each plant, a minimum of 150 root intersections were considered. At the end of the experiment (week 15), the root length of the *H. brasiliensis* plantlets was measured with a ruler before staining.

Enzyme histochemical staining of the AMF mycelium

The proportion of metabolically active hyphae was determined by measuring the activity of the succinate dehydrogenase enzyme (EC 1.3.99.1) -SDH (Saito 1995; Vierheilig et al. 2005), after 7, 11, and 15 weeks of growth in the HC. The phosphate metabolism was estimated by determining the activity of the alkaline phosphatase (ALP) enzyme (EC 3.1.3.1; van Aarle et al. 2002; Zocco et al. 2011) at the end of the experiment (week 15). The mycelium of *R. irregularis* in the HC of each system was extracted following solubilization of the medium with sodium citrate buffer (Doner and Becard 1991). The hyphae were rinsed with deionized water to remove the buffer and then stained for detection of the enzymatic activities. For the SDH activity, the samples were incubated overnight in the staining solution of 0.25 M sodium succinate, 0.05 M Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$ and 1 mg ml^{-1} Nitrotetrazolium blue chloride (Sigma-Aldrich, St. Louis, USA; Saito 1995) at room temperature in the dark (Zocco et al. 2011). For the ALP activity, a solution of 0.1 M Tris-HCl buffer (pH 8.5) was mixed with 1.1 mg $ml^{-1}$of alpha-Naphthyl phosphate Na salt (Sigma-Aldrich, St. Louis, USA) and 1.0 mg ml^{-1} fast blue RR salt (Sigma-Aldrich, St. Louis, USA). The samples were incubated 90 min at room temperature in the dark (Zocco et al. 2011).

After enzymatic staining, the samples for SDH and ALP activities were washed with deionized water for 15 min, counterstained with acid fuchsin (0.1% w/v acid fuchsin/lactic acid) for 15 min, and finally transferred to lactoglycerol (1:2:1 v:v:v lactic acid:glycerol:water) for de-staining and storage (Zocco et al. 2011). For the microscopic observations, the samples were mounted on microscope slides with lactoglycerol. Formazan (for SDH) or Fast blue RR granular precipitation (for ALP) was assessed with a compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) at x500 magnification. For each sample, at least 150 hyphal intersections were observed following the magnified intersects method (McGonigle et al. 1990). Intersections of hyphae were classified as active or non-active according to the presence or absence of granular precipitates, and the percentage of SDH and ALP active hyphae was determined.

Statistical analyses

Data analysis was performed with the statistical software Statistica® for Windows (StatSoft, 2001). For the percentage of total, arbuscules and intraradical spores/vesicles root colonization, and the percentage of SDH and ALP active hyphae, the values were arcsine $\sqrt{(x/100)}$ transformed before statistical analysis. After verification of the normal distribution, homogeneity of variances and residuals' independency of the data, two or three-way ANOVA ($P \leq 0.05$) was applied. The Tukey honest significant difference (HSD) test was used to identify the significant differences ($P \leq 0.05$).

Results

Root growth

At the start of the experiment, the *H. brasiliensis* plantlets presented the typical hierarchical root structure described by Le Roux and Pagès (1994) with a main taproot and five to ten early secondary roots (ESR) formed at the base of the shoot (Fig. 24). For the plantlets of the +AC-P and -AC-P treatments, the taproot developed until the end of the experiment, and several secondary roots of the acropetal sequence (SRAS) were produced 7 to 8 weeks after the transfer of the plantlets in the systems (Fig. 24). No new ESR were noticed, and the pre-existing ones had limited growth (i.e. ESR elongation was observed only in some plantlets grown in the presence of AC, see below).

Similarly, in the pruned plantlets of the +AC+P and –AC+P treatments, no new ESR were produced. A rapid regeneration of the taproot was observed one to two weeks after pruning, with a new taproot that emerged at the wound site and continued to grow until the end of the experiment. The new taproot also produced several SRAS from week 7 to 8, until the end of the experiment.

No significant differences were noted in the total root length of the intact plantlets (-P) and the plantlets with excised taproot (+P) after 15 weeks of growth (Table 6). However, the total root length of the plantlets was significantly higher after 15 weeks of growth in the presence of AC added to the MSR medium as compared to the plantlets grown in the absence of AC (Table 6). No significant effect of the interaction between the AC and P treatments was observed on the root length of the *H. brasiliensis* plantlets.

AMF root colonization

Whatever the treatment, the first traces of root colonization were observed 7 weeks after placing the *H. brasiliensis* plantlets in the mycelium network of *R. irregularis*. Intraradical hyphae and spores/vesicles were observed in the cortex of some ESR, and in the distal part of the taproot. The first arbuscules were recorded at week 9 in the SRAS.

Most of the ESR roots (that were present in the plantlets at transfer to the MDP *in vitro* culture systems) had limited or no growth. Nevertheless, elongation of the root apex of a few millimeters was observed in the ESR of some plantlets in the presence of AC (+AC+P and +AC–P treatments). In that case, the formation of arbuscules in the new tissues (at week 9) was also noticed.

Significantly higher values of total root colonization were obtained with the addition of AC (P <0.0001) and with time (P <0.0001). In contrast, the plantlets in the taproot pruning treatments showed significantly lower values (P <0.0001). Significant 2-way interaction effects were observed between addition of AC or pruning and time (AC x T, P <0.0001 and P x T, P <0.05, respectively). Similarly, a significant 3-way interaction effect between addition of AC, pruning of the taproot and the different measurements over time was observed for the total root colonization (AC x P x T; P <0.05). At the end of the experiment (i.e. after 15 weeks of culture in the ERM network), the highest values of total root colonization (62.5% ± 3.0) were observed in the +AC-P treatment, and the lowest (25.0 % ± 1.8) in the -AC+P treatment (Fig. 25 A). From week 13, the plantlets in the AC treatments showed higher total root colonization as compared to those grown in the MSR medium devoid of AC. Furthermore, the total root colonization of the plantlets of the +AC-P treatment became significantly higher as compared to the plantlets of the +AC+P treatment.

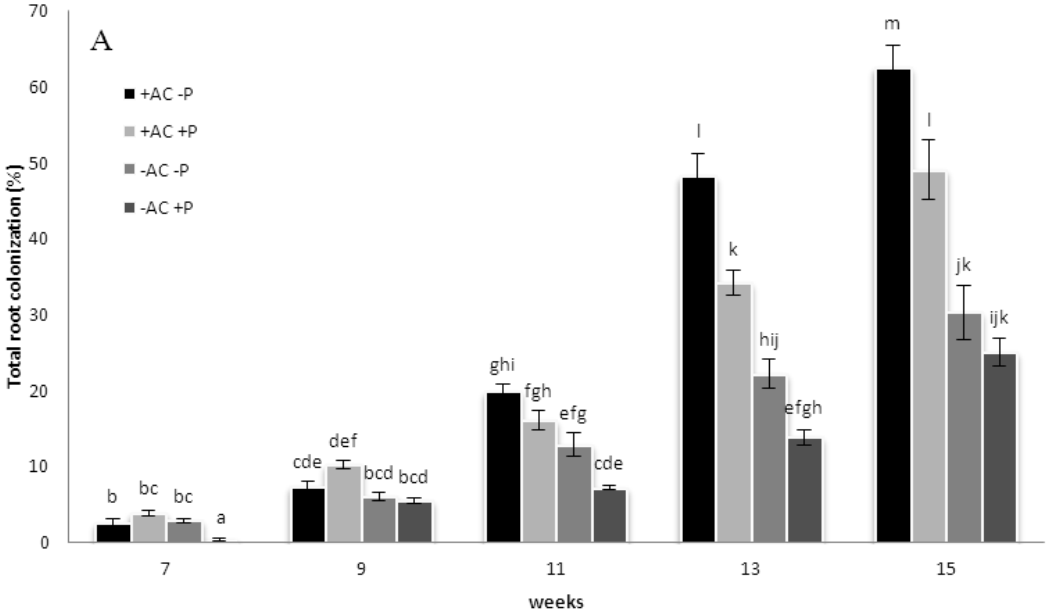
Higher percentages of arbuscules were observed in the presence of AC (P<0.0001) and with time (P<0.0001). The pruning of the taproot did not significantly impact this parameter. Two-way interaction effects were observed between the addition of AC or pruning and time (AC x T, P <0.0001 and P x T, P <0.0001, respectively). Similarly, a significant 3-way interaction effect was observed (AC x P x T; P <0.05). The formation of arbuscules started at week 9, and significantly increased thereafter for all the treatments (post-hoc analysis, Fig. 25 B). At week 11, the abundance of arbuscules in treatment +AC+P was significantly higher than in the other treatments, reaching values of 10.2% ± 1.1. From week 13 to 15, the abundance of arbuscules in the roots of the plantlets grown in the presence of AC was significantly higher as compared to those of the -AC treatments. At week 15, the plantlets of the +AC-P treatments had a significantly higher percentages of arbuscules than the other treatments.

The abundance of intraradical spores/vesicles was higher in the presence of AC in the MSR medium (P<0.0001) and with time (P<0.0001). The pruning of the taproot also significantly impacted this parameter (P<0.0001). Figure 25 C shows the percentages of intraradical spores/vesicles obtained for the different treatments. No significant 3-way interaction was observed between the different factors. Nevertheless, significant effects for all three 2-way interactions were observed: AC x P (P <0.05); AC x T (P <0.0001) and P x T (P <0.05). Post-hoc analyses of these interactions showed that the percentages of intraradical spores/vesicles in the plantlets grown in presence of AC were

significantly higher than in the plantlets grown without AC addition for both -P and +P treatments. In contrast, no significant differences were observed between the +AC-P and +AC+P treatments. The pruning of the taproot significantly limited the percentage of intraradical spores/vesicles in the treatments that did not receive AC. Significant higher values of intraradical spores/vesicles colonization were observed from week 9 to 15 for the plantlets grown in presence of AC, in comparison with the plantlets of the -AC treatments. The taproot pruning did not significantly affect the percentage of intraradical spores/vesicles from week 7 to 11 and at week 15. However, at week 13, the abundance of intraradical spores/vesicles was significantly lower in the plantlets with excised taproot.

Enzymatic activity of the AMF mycelium

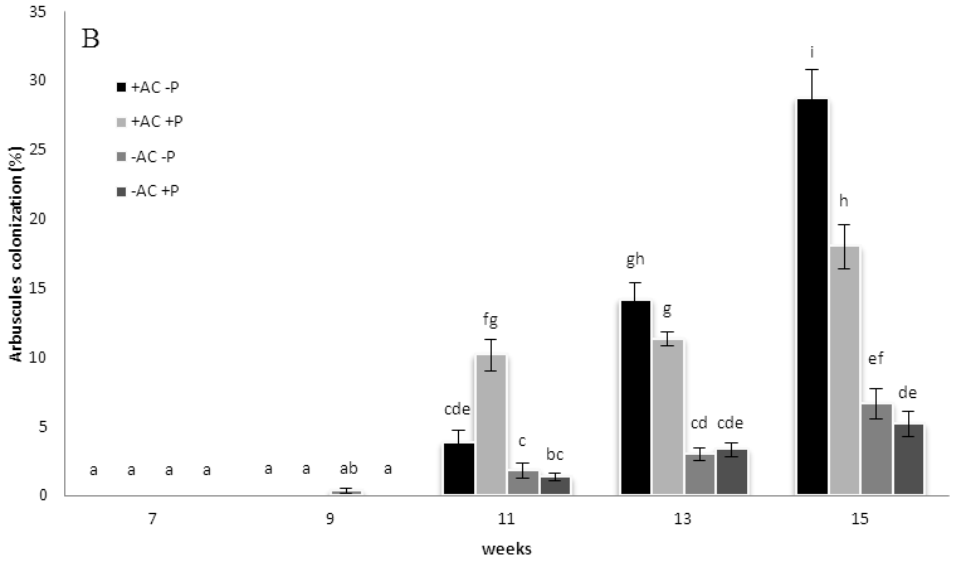
The mycelium viability measured as SDH enzymatic activity, was not significantly affected by the addition of AC or the pruning of the taproot (Table 6). In contrast, the proportion of SDH active hyphae significantly decreased with time. No significant interactions between the factors (2 or 3-way ANOVA) were found for this parameter. The alkaline phosphatase (ALP) enzyme activity measured at the end of the experiment was not impacted by the addition of AC, the pruning of the taproot, or their interaction (Table 6).



Histograms represent means of four replicates ±SE. Bars followed by different letters differ significantly following post-hoc analyses of the significant interaction AC x P x T (Three-way ANOVA, $P \leq 0.05$; Tukey's HSD test, $P \leq 0.05$).

Data were recorded after 7, 9, 11, 13 and 15 weeks of growth in contact with the extraradical mycelium of *R. irregularis*.

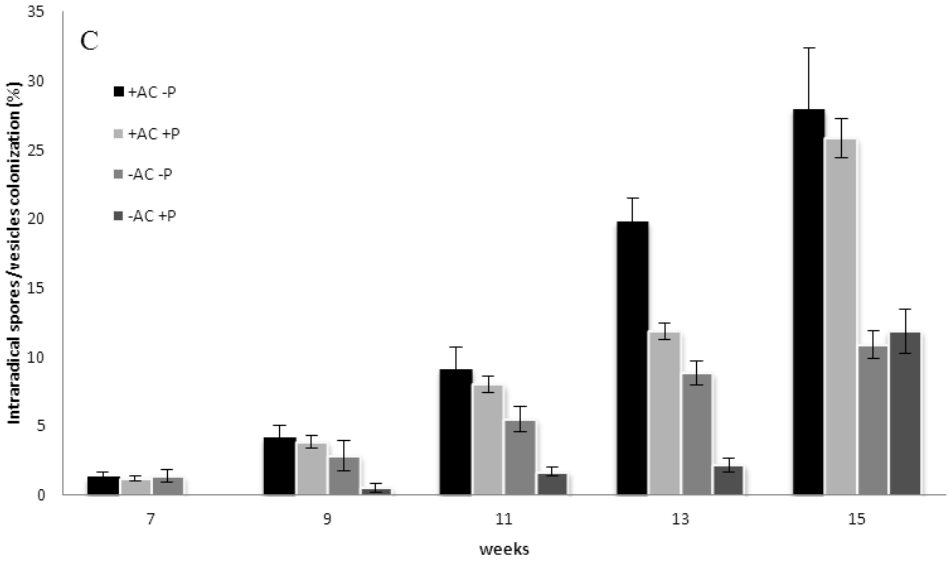
Figure 25. **A.** Percentages of total root colonization of *H. brasiliensis* clone PB 260 *in vitro* in presence (+AC) or absence (-AC) of activated charcoal and pruning (+P) or not (-P) of the taproot.



Histograms represent means of four replicates ±SE. Bars followed by different letters differ significantly following post-hoc analyses of the significant interaction AC x P x T (Three-way ANOVA, $P \leq 0.05$; Tukey's HSD test, $P \leq 0.05$).

Data were recorded after 7, 9, 11, 13 and 15 weeks of growth in contact with the extraradical mycelium of *R. irregularis*.

Figure 25. **B.** Percentages of arbuscules colonization of *H. brasiliensis* clone PB 260 *in vitro* in presence (+AC) or absence (-AC) of activated charcoal and pruning (+P) or not (-P) of the taproot.



Histograms represent means of four replicates ±SE (Three-way ANOVA, $P \leq 0.05$).

Data were recorded after 7, 9, 11, 13 and 15 weeks of growth in contact with the extraradical mycelium of *R. irregularis*.

Figure 25. **C.** Percentages of intraradical spores/vesicles root colonization of *H. brasiliensis* clone PB 260 *in vitro* in presence (+AC) or absence (-AC) of activated charcoal and pruning (+P) or not (-P) of the taproot.

Table 6. Effects of the presence (+AC) or absence (-AC) of activated charcoal, in combination with the pruning (+P) or not (-P) of the taproot of *H. brasiliensis* clone PB 260 plantlets, grown in contact with the extraradical mycelium (ERM) network of *R. irregularis* MUCL 41833 for 15 weeks.

The total root length (cm) of the plantlets, and the percentage of alkaline phosphatase (ALP) active hyphae in the ERM network of the fungus, were recorded at the end of the experiment (week 15). The percentage of succinate dehydrogenase (SDH) active hyphae of *R. irregularis* was recorded at weeks (T) 7, 11, and 15.

Activated Charcoal	Taproot	Time (weeks)	SDH (%)	ALP (%)	Total root length (cm)
Present (+AC)	Present (-P)	7	65,7 ± 2,3	-	-
		11	61,2 ± 4,6	-	-
		15	52,3 ± 1,5	53,8 ± 4,5	123,3 ± 5,3
	Pruned (+P)	7	67,1 ± 3,3	-	-
		11	60,7 ± 5,9	-	-
		15	52,8 ± 1,4	50,6 ± 1,6	109,4 ± 7,4
Absent (-AC)	Present (-P)	7	63,4 ± 0,9	-	-
		11	58,1 ± 2,7	-	-
		15	54,2 ± 2,5	57,7 ± 5,8	72,3 ± 7,0
	Pruned (+P)	7	68,8 ± 1,4	-	-
		11	60,1 ± 4,2	-	-
		15	47,9 ± 3,2	63,0 ± 5,7	68,8 ± 7,0
Significance of treatment					
AC			NS	NS	<0.0001
P			NS	NS	NS
T			<0.0001	-	-
AC x P			NS	NS	NS
AC x T			NS	-	-
P x T			NS	-	-
AC x P x T			NS	-	-

NS: Not significant
-: Not measured

Data represent means +/- SE of four replicates. SDH: Three-way ANOVA, $P \leq 0.05$. ALP and total root length: Two-way ANOVA, $P \leq 0.05$

Discussion

Arbuscular mycorrhizal fungi are naturally present in the roots of the rubber tree (Feldmann 1994; Schwob et al. 1999). These soil microorganisms can improve the growth of young *H. brasiliensis* seedlings grown in the nursery (Ikram et al. 1992; Feldmann 1994). Furthermore, the inoculation of several micropropagated plant species with AMF most often resulted in improved plant resistance to biotic or abiotic stresses during acclimatization (Vestberg and Cassells 2009).

The association of *H. brasiliensis* plantlets with *R. irregularis* under *in vitro* autotrophic culture conditions was recently achieved (Sosa-Rodriguez et al. 2013a). Intraradical colonization with the production of arbuscules and intraradical spores/vesicles was observed. However, root colonization was detected only after 7 weeks of *in vitro* culture. This delay may be attributed to the slow emission of new roots, since the presence of secondary and tertiary roots (without secondary growth) has been reported to be important for AMF root colonization of several plants species (Hepper 1981; Hepper 1985; Afek et al. 1990; Amijee et al. 1993; Brundrett 2002; Guo et al. 2008). In addition, the exudation of inhibitory molecules by the plant roots that may negatively impact AMF has also been suggested for several plant species (Vierheilig and Bago 2005; Stinson et al. 2006) among which *H. brasiliensis* (Sosa-Rodriguez et al. 2013b).

Here, the combination of AC with the pruning of the taproot was tested on root growth and subsequent colonization of *H. brasiliensis*. At the end of the experiment, the plantlets grown in the presence of AC had longer roots, higher percentages of total root colonization, arbuscules and intraradical spores/vesicles than the plantlets of the -AC treatments. In contrast, pruning of the taproot did not influence the root growth or the percentage of arbuscules, while lower percentages of total root colonization and intraradical spore/vesicles were found in the pruned plantlets. The metabolic activity of the ERM network of *R. irregularis* (measured as the SDH and ALP enzymatic activities) was not affected by the AC or P treatments.

Activated charcoal has been reported to stimulate the rooting process of herbaceous and woody plants from different taxonomic groups (Pan and Staden 1998). It was also described as a compound suitable to sequestrate organic substances that may be harmful to the plants (Thomas 2008). In the present study, we observed higher values of total root length after addition of AC to the MSR medium. This stimulation was observed in both normal and pruned plantlets. The increased root length was probably related with

the elongation of the taproot apex and the production of more SRAS in the presence of AC (although the number of roots was not quantified during these experiments).

Interestingly, the addition of AC to the MSR medium did not accelerate the formation of new SRAS or ESR (which were produced only 7 to 8 weeks after the transfer in the MDP *in vitro* culture systems). These results suggested that the addition of AC to the MSR medium stimulated the root growth (i.e. the root elongation) of the rubber plantlets (possibly via the sequestration of exogenous compounds impacting the root growth) but did not influence the root initiation (i.e. the activation of the meristems that originate new roots).

The impact of AC on the growth and development of mycorrhizal fungi has also been demonstrated. Herrmann et al. (2004) observed that AC improved the association between the ECM fungus *Piloderma croceum* and oak micro-cuttings *in vitro*. Favorable effects of AC on AMF spore germination (Daniels and Trappe 1980), and stimulation of root colonization in the presence of biochar (the charcoal variant used in soils experiments) were also reported (Rillig et al. 2010; Solaiman et al. 2010; Elmer and Pignatello 2011). In contrast, Weißhuhn and Prati (2009) observed reduced root colonization following AC addition. Wurst et al. (2010) suggested that charcoal could interrupt the communication between the host plant and the AMF by adsorbing signaling compounds such as strigolactones, but this interference remains to be demonstrated (Akiyama et al. 2005; Lehmann et al. 2011).

The positive effect of AC on root colonization (i.e. total colonization, arbuscules and intraradical spores/vesicles production) observed in our experiments suggested that AC may impact AMF directly by stimulating hyphal growth, or indirectly by improving the root growth. In fact, we observed enhanced root elongation after the addition of AC to the culture medium. The need of new roots to favor root colonization by AMF has been reported (see below). Whatever the mechanism involved, the consequence of this stimulation would be the improvement of the interaction between both organisms.

It is also possible that AC addition had an indirect effect on AMF root colonization by adsorbing root exudates that could impair the mycorrhization process (Warnock et al. 2007; Rillig et al. 2010; Solaiman et al. 2010; Elmer and Pignatello 2011). *H. brasiliensis* latex is known to possess antimicrobial and antifungal properties and several compounds related to its biocidal capacities have been identified (Martin 1991; Jekel et al. 2005;

Kanokwiroon et al. 2008; Taira et al. 2005). For instance, a small (4.7 KDa) cysteine-rich protein that is a major component of the latex of natural rubber, hevein, was reported by Van Parijs et al. (1991) to inhibit the mycelial growth of *Botrytis cinerea, Septoria nodorum, Trichoderma hamatum* and *Phycomyces blakesleeanus*.

Therefore, it is not excluded that specific antifungal compounds such as hevein, chitinases, or other antimicrobial peptides (see Odintsova and Egorov 2012 for a comprehensive review), could be exuded in vitro by the roots of *H. brasiliensis*, impacting (at least transiently) the development of the AMF and the root colonization. Indeed, the reduction on root colonization of neighbouring plants in presence of *H. brasiliensis* has been described recently (Sosa-Rodriguez et al. 2013b).

Interestingly, the abundance of arbuscules was enhanced in presence of AC, while the taproot pruning had no impact on this parameter. In addition, the enzymatic activities (ALP and SDH) were not affected by the treatments. These results suggested that neither the presence of AC nor the taproot pruning (and/or their combination), disturbed the intraradical root colonization or the phosphate metabolism and the viability of the ERM network of *R. irregularis*. Nevertheless, the pruning treatment resulted in lower total root colonization and reduced percentages of intraradical spores/vesicles, especially in the absence of AC, suggesting that the stimulation of the root colonization by AC would be better expressed in an intact root system (i.e. without any modification of the root morphology).

The pruning of the taproot was applied to induce the production of new roots by the rubber plantlets. After pruning, a new taproot regenerated from the site of wounding, and new SRAS were produced. However, at the end of the experiments, we obtained similar total root length in both pruned (+P) and intact plantlets (-P), without any influence on the emission of new ESR. Thaler and Pagès (1997) observed the same response after the excision of the taproot of rubber seedlings growing in a soil substrate.

We also observed that whatever the treatment (i.e. addition of AC or taproot pruning), the root colonization only started after seven weeks of contact between *H. brasiliensis* and the ERM network of *R. irregularis*, when the formation of novel roots and/or their elongation were observed. Thus, the emission of new lateral roots, and possibly the root anatomy (i.e. presence of secondary growth and suberization), seemed important for establishing the mycorrhizal symbiosis between *H. brasiliensis* and *R. irregularis*. Hepper (1981) observed that secondary roots of *Trifolium parviflorum* newly

produced *in vitro*, were more susceptible to the colonization by the AMF *Funneliformis mosseae* than pre-existing older roots.

Recently, Putranto et al. (2012) compared the anatomy of the taproot and the lateral roots of *H. brasiliensis* plantlets produced *in vitro*. The taproot showed primary and secondary growth, while lateral roots only presented primary growth. They also found a differential expression of genes between the taproot and the lateral roots, and suggested that each type of roots could have specialized functions, with secondary and tertiary roots mainly involved in nutrients uptake. In our study, we observed that these roots were also the more susceptible to root colonization. Interestingly, as the genes expression of the lateral roots appeared to be highly controlled by the plant (Putranto et al. 2012), a regulation of the interaction with AMF may be hypothesized. The expression of plant genes involved in the accumulation and/or exudation of secondary metabolites by the rubber plant roots should be explored, in order to understand other factors that could influence the early mycorrhization of *H. brasiliensis*.

The addition of AC to the culture medium had a positive influence on root growth and colonization of *H. brasiliensis*. However, neither AC presence nor taproot pruning accelerated the production of new roots (SRAS or ESR) or root colonization. Furthermore, the observed increase in the total root colonization following the addition of AC to the MSR medium was statistically significant after 13 weeks of culture. Focus on the beneficial role of AC on root growth, and especially root colonization could thus allow further improvement of the mycorrhization of *H. brasiliensis.* In this respect, it seems particularly important to establish which root exudates of *H. brasiliensis* could putatively limit AMF colonization. Isolation of putative antifungal molecules present in the root exudates and characterization of their functional roles could help to improve the *in vitro* mycorrhization of rubber plantlets.

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Chapter 4

Hevea brasiliensis and *Urtica dioica* impact the *in vitro* mycorrhization of neighbouring *Medicago truncatula* seedlings

Preface

Hevea brasiliensis plantlets were successfully mycorrhized using the MDP *in vitro* culture system. Nevertheless, specific culture conditions were necessary to stimulate root growth and colonization. The combination of CO_2 and MES (**Chapter 2**) and the addition of activated charcoal (**Chapter 3**) enhanced the root colonization by the AMF. However, the period needed for root colonization and production of arbuscules remained too long (approx. 9 weeks). Therefore, in order to explain this response, two hypotheses were proposed: (1) *H. brasiliensis* plantlets produce exudates containing antifungal compounds (2) The exudation of such compounds may disrupt the root colonization of a highly mycotrophic plant (*M. truncatula*) by the ERM network of *R. irregularis*. *Urtica dioica* seedlings were used as a positive control, since this plant is largely known as non-host for AMF. *U. dioica* is also reported to exudate UDA, an antifungal protein (Vierheilig et al. 1996). The results presented in this chapter suggested that *H. brasiliensis* (and *U. dioica*) produced exudates that impaired the *in vitro* colonization of *M. truncatula*. We also hypothesized that such exudates may be responsible for the delay in the *in vitro* colonization of the rubber tree plantlets.

Hevea brasiliensis and *Urtica dioica* impact the *in vitro* mycorrhization of neighbouring *Medicago truncatula* seedlings

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Abstract

Hevea brasiliensis is a mycotrophic tree for which root colonization by arbuscular mycorrhizal fungi (AMF) under *in vitro* or pot culture conditions can take several weeks. The reason for this slow colonization is still unknown, but the exudation of antifungal compounds such as hevein by the roots may be one of the causes. Here, the root colonization of *Medicago truncatula*, a highly mycotrophic plant, was assessed after 12 days of growth in the extraradical mycelium network of the AMF *Rhizophagus irregularis* in close vicinity of *H. brasiliensis* plantlets or *Urtica dioica* seedlings (also known to synthesize antifungal compounds of the hevein family). We hypothesized that a negative impact on the root colonization of a *M. truncatula* seedling developing close to *H. brasiliensis* and *U. dioica* may give indirect proofs for the exudation of inhibitory molecules. The percentages of total root colonization of *M. truncatula* were 30.1% lower in the presence of *H. brasiliensis* than in the control plants, and 29.1% lower in presence of *U. dioica*. The abundance of arbuscules in the roots of *M. truncatula* was also lower in plants grown in presence of *H. brasiliensis* plantlets than in the control plants. Similarly, the succinate dehydrogenase and the phosphatase activities measured in the extraradical mycelium of *R. irregularis* were significantly lower in the presence of both plants, compared with the controls. No root colonization was observed in *H. brasiliensis* and *U. dioica* within the time-frame of the experiments. The low root colonization of *M. truncatula* when grown in the presence of rubber or stinging nettle suggested the exudation of diffusible molecules which could also explain the delayed root colonization of *H. brasiliensis* and the absence of colonization of *U. dioica.*

Key Words arbuscular mycorrhizal fungi • *Hevea brasiliensis* • *Urtica dioica* • *Medicago truncatula* • *Rhizophagus irregularis* • extraradical mycelium network

Introduction

Arbuscular mycorrhizal fungi (AMF) are symbiotic soil microorganisms that colonize the roots of nearly 70% of vascular plants (Brundrett 2009). In the roots, these fungi develop intercellular and intracellular hyphae (Bonfante and Genre 2010) that differentiate within cells into arbuscules, the typical structures involved in the bi-directional transport of carbohydrates from the plant to the fungus, and minerals (e.g. phosphorus or nitrogen) from the fungus to the plant. This active exchange is essential to the development of the obligate fungal symbiont and the improvement of plant growth. AMF also favor plant resistance to abiotic and biotic constraints and influence plant diversity and ecosystem productivity (van der Heijden et al. 1998; Smith and Read 2008).

Although symbiosis with AMF is reported in the vast majority of land plants, different families and species have been described as non-host (Brundrett 2002; Brundrett 2009). Several factors have been suggested to explain the absence of colonization: (1) the plant is unable to produce essential signals for triggering the different steps leading to root colonization (Giovannetti and Sbrana 1998); (2) the plant cannot recognize fungal signals sent during the pre-symbiotic stage (Vierheilig and Bago 2005; Parniske 2008) (3) the plant has barriers against the mycorrhizal colonization that are intrinsic to the roots (i.e. related to physiological characteristics of the root cortex or epidermis; Fontenla et al. 1999); (4) the plant produces and exudates inhibitory or antifungal compounds (Vierheilig et al. 1996; Vierheilig and Bago 2005; Stinson et al. 2006) that impair the fungal growth.

A number of studies have reported on the impact of plant inhibitory signals and chemical compounds on different steps of the AMF development (from spore germination to root colonization and arbuscules formation) under *in vitro*, (Vierheilig and Bago 2005), greenhouse (Yun and Choi 2002; Stinson et al. 2006) and field (Barto et al. 2011) conditions. Interestingly, root exudates or extracts from non-host plants, such as the stinging nettle (*Urtica dioica* L.) have also been shown to influence the root colonization of neighbouring mycotrophic plants (Vierheilig et al. 1995; Vierheilig et al. 1996; Vierheilig et al. 2003; Fontenla et al. 1999).

U. dioica has been classified as a non-host plant, since colonization of its roots by AMF has never been observed (Renker et al. 2005; Fontenla et al. 1999; Vierheilig et al. 1996; Helgason et al. 2007; Bainard et al. 2011). *U. dioica* plants produce and accumulate a monomeric 8 kDa protein called *Urtica dioica* agglutinin (UDA) in their roots and rhizomes (Peumans et al. 1984;

Broekaert et al. 1989), which was shown to inhibit the mycelial growth of several saprophytic and pathogenic fungi such as *Botrytis cinerea, Septoria nodorum, Trichoderma hamatum* and *Phycomyces blakesleeanus* (Van Parijs et al. 1991). The antifungal properties of UDA have been attributed to its capacity to bind chitin oligosaccharides, which are main components of the fungal wall (Broekaert et al. 1989). UDA has also been reported to inhibit the growth of hyphae arising from germinated spores of the AMF *Fulleniformis mosseae* (formerly *Glomus mosseae*) under *in vitro* culture conditions as well as the root colonization of *Glycine max* (Vierheilig et al. 1996) and *Pisum sativum* (Fontenla et al. 1999).

Interestingly, the amino acid sequence of UDA presents high homology with hevein, a smaller protein that is a major component of the latex of natural rubber, *Hevea brasiliensis* Müll. Arg. (Van Parijs et al. 1991; Gidrol et al. 1994). Antimicrobial properties of latex have been observed *in vitro* (Kanokwiroon et al. 2008) and the capacity of purified hevein to inhibit the mycelial growth of *B. cinerea, S. nodorum, T. hamatum* and *P. blakesleeanus* was reported by Van Parijs et al. (1991).

H. brasiliensis is a mycotrophic plant (Ikram et al. 1992; Ikram et al. 1993). However, its colonization by AMF under greenhouse conditions may take several weeks (Schwob et al. 2000). Root colonization of young plantlets under *in vitro* culture conditions growth in a dense extraradical mycelium network also took a long time, as the plantlets were colonized only after a period of at least seven weeks (Sosa-Rodriguez et al. 2013a). The reasons for this delay are still unknown, but the exudation of hevein (or another antifungal compound) from roots could be a realistic explanation. As reported for *U. dioica* (Vierheilig et al. 1996; Fontenla et al. 1999), studying the colonization of a highly mycotrophic plant developing in close vicinity of *H. brasiliensis* may thus indirectly support this hypothesis. Here, we used the mycelium donor plant (MDP) *in vitro* culture system developed by Voets et al. (2009) to investigate the effects of *H. brasiliensis* or *U. dioica* on the root colonization of *Medicago truncatula* by *Rhizophagus irregularis* MUCL 41833 and the metabolic activity in the extraradical mycelium of the AMF.

Materials and methods

Urtica dioica *seedlings*

Seeds of stinging nettle, *U. dioica* L., (Herbiseed, Berkshire, UK) were surface-sterilized by immersion in ethanol (70%) for 5 min followed by rinsing in deionized sterile water for 10 min and immersion in sodium hypochlorite (8% active chloride) for 15 min. The seeds were finally rinsed three times in deionized sterile water and germinated in 107x94x65 mm sterile containers (Duchefa Biochemie B.V., Haarlem, the Netherlands) filled with 100 ml of modified Strullu-Romand (MSR) medium (Declerck et al. 1998) lacking sucrose and vitamins, and solidified with 3 g l^{-1} Phytagel™ (Sigma-Aldrich, St. Louis, USA). Thirty seeds were plated in each container. The containers were placed in a growth chamber under controlled conditions (22°C/18°C day/night, 70% relative humidity, 16 hd^{-1} photoperiod and a photosynthetic photon flux of 225 μmol m^{-2} s^{-1}). Seeds germinated within 8-10 days and seedlings were ready to use after 20 days, when the length of the shoots was approximately 20 mm.

Hevea brasiliensis *plantlets*

Twelve-week-old plantlets of *H. brasiliensis* Müll. Arg. clone PB 260 (Prang Besar, Malaysia) were produced by somatic embryogenesis (MFP MICHELIN & CIRAD, Clermont-Ferrand and Montpellier, France). Before transfer to the MDP *in vitro* culture systems (see below), the plantlets were grown under mixotrophic culture conditions inside glass tubes (25 mm diameter x 200 mm height) filled with 30 ml of an agar-gelled culture medium adapted to the embryo germination and growth of *H. brasiliensis*. This medium contained 234 mM of sucrose, but no growth regulators (Lardet et al. 2007). The lid of the tubes had a porous filter (3M™ Micropore™Tape) that allowed the gas exchange, maintaining the sterility in the tubes. The plantlets were placed in a growth chamber set at 27 °C, with a 12 hd^{-1} photoperiod, and a photosynthetic photon flux of 60 μmol m^{-2} s^{-1}. At the time of the experiment, the plantlets had 1-3 leaves, 4-6 cm stem height, 4-6 cm taproot length and 5-10 lateral roots of 1-3 cm length.

Medicago truncatula *seedlings*

Seeds of barrel medic, *M. truncatula* L., cv. Jemalong strain A17 (SARDI, Adelaide, Australia), were surface-sterilized by immersion in sodium hypochlorite (8% active chloride) for 15 min followed by rinsing three times in deionized sterile water. Seeds were germinated in Petri plates (90 mm diameter) filled with 30 ml of MSR medium. Fifteen seeds were plated per

Petri plate and further incubated at 18-22°C in the dark. Seeds germinated within 1-2 days and were ready to use after 4 days when the length of the shoots was approximately 20 mm.

Arbuscular mycorrhizal fungus

Rhizophagus irregularis (Błaszk, Wubet, Renker & Buscot) C. Walker & Schuessler (2010) [as 'irregulare'] MUCL 41833 (Synonymy: *Glomus irregulare*) was purchased from the Glomeromycota *in vitro* collection (GINCO www.mycorrhiza.be/ginco-bel). This AMF was initially associated and sub-cultured using the Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 (for details, see Cranenbrouck et al. 2005). Afterwards, the strain was mass-produced in association with *M. truncatula* in the Half-Closed Arbuscular Mycorrhizal–Plant (HAM–P) *in vitro* culture system (Voets et al. 2005). Several thousands of spores were produced in the hyphal compartment (HC) of this system within a period of 10 weeks.

Experimental set-up

Experiment 1: *In vitro* mycorrhization of *Medicago truncatula* in presence of *Urtica dioica*

Mycelium Donor Plant (MDP) *in vitro* culture systems were established associating a *M. truncatula* plantlet with spores of *R. irregularis* MUCL 41833 to produce an extraradical mycelium (ERM) network (for details, see Voets et al. 2009). Briefly, each MDP *in vitro* culture system consisted of a bi-compartmented Petri plate of 90 mm diameter allowing the physical separation of a root compartment (RC) from a hyphal compartment (HC; Fig. 26). Both compartments were filled with 20 ml of MSR medium without sucrose and vitamins. The *M. truncatula* seedling was transferred to the RC and inoculated with a plug of gel of 0.4 cm^3 containing approximately 100 spores of *R. irregularis*. The MDP *in vitro* culture systems were placed in a growth chamber under controlled conditions (22°C/18°C day/night temperature, 70% relative humidity, 16 hd^{-1} photoperiod and a photosynthetic photon flux of 225 μmol m^{-2} s^{-1}). Starting from week 3, 10 ml MSR medium lacking sucrose and vitamins was added weekly to the RC (Voets et al. 2009).

After a period of 8 weeks, hyphae crossed the partition wall between the two compartments and spread on the HC. To assure the homogeneous growth of the ERM into the HC, the medium of this compartment was almost entirely removed, but a band of medium (approximately 2 mm width) parallel to the partition wall was kept to maintain the mycelium connection between the

RC and the HC. Twenty-five ml fresh MSR medium without sucrose and vitamins was then added to the HC. Two weeks later, the systems in which the mycelium had covered the complete surface of the HC were selected for the experiment.

In half of the selected MDP *in vitro* culture systems, four *U. dioica* seedlings (UD treatment) were plated on the surface of the HC (Fig. 26 A). The shoot of these plants remained inside the HC. In the other half, no *U. dioica* seedlings were plated in the ERM of the HC (control treatment – CT-UD). The lids of the systems were replaced by new ones with two holes of 0.6 mm diameter covered by a sterilized (121°C for 15 min.) double-layer white strip of 20x20 mm (3M™ Micropore™ Tape) allowing gas exchanges and thus photosynthesis of *U. dioica*, but avoiding microbial contamination (Fig. 26 B). One week later, a 4-day-old *M. truncatula* seedling was introduced in the HC of each MDP *in vitro* culture system with the roots in contact with the ERM and the shoot extending outside the system (Fig. 26). The *M. truncatula* seedlings were grown for 4, 8 and 12 days in the presence (UD) or the absence (CT-UD) of *U. dioica*. The systems were placed in the growth chamber under the same growth conditions as described above. Four replicates (i.e. seedlings of *M. truncatula*) per treatment were collected at each time.

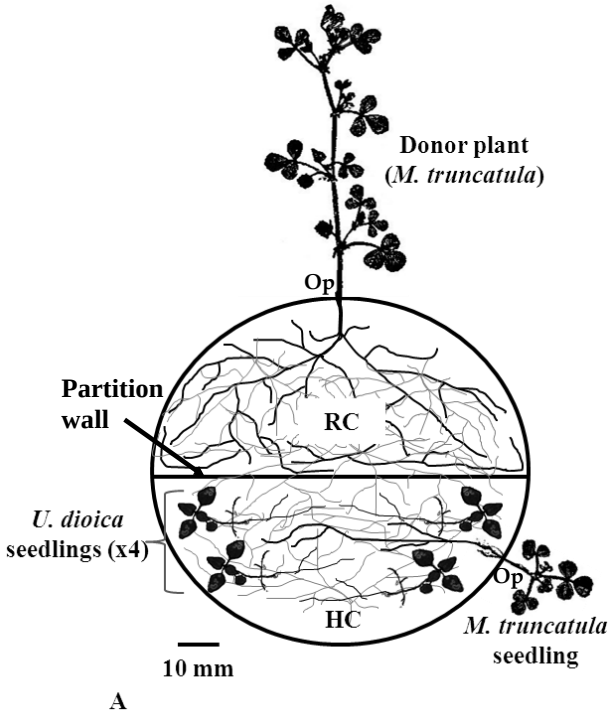
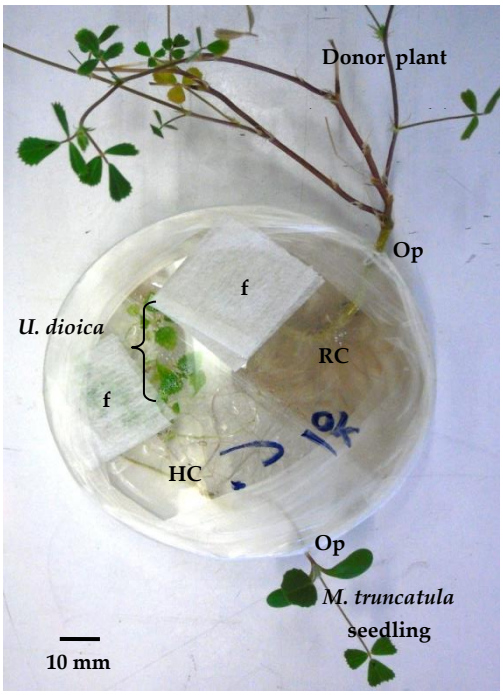


Figure 26. **A.** Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) to study the impact of *Urtica dioica* on the mycorrhization of *Medicago truncatula* by *Rhizophagus irregularis* MUCL 41833.
Schematic representation of the system showing the disposition of the *U. dioica* plants, the root compartment (RC) with the opening (Op) of approx. 2mm diam. made to allow the development of the plant shoot outside the Petri plate, the partition wall (separation between the compartments), and the hyphal compartment (HC).



B

Figure 26. **B.** Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) to study the impact of *Urtica dioica* on the mycorrhization of *Medicago truncatula* by *Rhizophagus irregularis* MUCL 41833.
Urtica dioica treatment (UD) system showing the disposition of the 3M™ Micropore™ Tape filters (f) on the system lid, the *M. truncatula* donor plant, and the *M. truncatula* test plant.

Experiment 2: *In vitro* mycorrhization of *Medicago truncatula* in presence of *Hevea brasiliensis*

The MDP *in vitro* culture system mentioned above was slightly modified following the methodology described by Sosa-Rodriguez et al. (2013a). Briefly, the bi-compartmented system was set up by placing a small Petri plate (55 mm diameter x 8 mm *height* – the RC) into a large Petri plate (*145 mm diameter x 20 mm height – the HC*; Fig. 27 A). The lid of the system was also modified with the addition of a screw cap tube (30 mm diameter x 35 mm length; Fig. 27 B) to facilitate the addition of medium. Starting from week 3, 10 ml MSR medium lacking sucrose and vitamins was added weekly to the RC (Sosa-Rodriguez et al. 2013a). The systems were gamma sterilized (25 KGy) before utilization. *M. truncatula* was used as donor plant and inoculated with spores of the AMF as described above. The systems were incubated during 10 weeks to obtain a profuse ERM network in the HC.

Only the systems in which the mycelium had covered 70% of the HC surface (approximately 75 cm^2) were used. In half of these MDP *in vitro* culture systems, a twelve-week-old *H. brasiliensis* plantlet (HB treatment) was placed in the HC with the roots in contact with the ERM network and the shoot extending outside the systems (Fig. 27), while in the other half, the HC remained free (control treatment – CT-HB). All the systems were then placed in a growth chamber under controlled conditions (26°C/23°C day/night temperature, 70% relative humidity, 12 hd^{-1} photoperiod and a photosynthetic photon flux of 60 µmol m^{-2} s^{-1}) adapted to the acclimatization of *H. brasiliensis* plantlets produced *in vitro*.

One week later, a 4-day-old *M. truncatula* seedling was introduced in the HC of each MDP *in vitro* culture system (next to the *H. brasiliensis* plantlet in the HB treatment), with the roots in contact with the ERM and the shoot extending outside the system (Fig. 27). The *M. truncatula* plantlets were grown for 12 days in the presence (HB) or the absence (CT-HB) of *H. brasiliensis*. During this period, the systems were placed in a growth chamber under controlled conditions (22°C/18°C day/night temperature, 70% relative humidity, 16 hd^{-1} photoperiod and a photosynthetic photon flux of 225 µmol m^{-2} s^{-1}). Four replicates (i.e. seedlings of *M. truncatula*) per treatment were collected after 12 days of contact with the ERM.

Harvest and data collection

To study the impact of *U. dioica* (Exp. 1) and *H. brasiliensis* (Exp. 2) on the root colonization of *M. truncatula,* the entire root systems of the *M. truncatula*

seedlings were collected and intraradical colonization estimated after 4, 8 and 12 days of contact with the ERM for *U. dioica*, and after 12 days for *H. brasiliensis*. The root systems of *U. dioica* and *H. brasiliensis* were also harvested. In parallel, culture medium of the HC containing the mycelium of *R. irregularis* was sampled in each system to evaluate the proportion of metabolically active hyphae by measuring the activity of the succinate dehydrogenase enzyme (SDH; Saito 1995; Vierheilig et al. 2005), and the phosphate metabolism by determining the activities of the alkaline phosphatase (ALP) and acid phosphatase (ACP) enzymes (van Aarle et al. 2002; Zocco et al. 2011). These analyses were done at day 4, 8 and 12 in Exp. 1 and day 12 in Exp. 2.

Root colonization

After sampling, the root length of the *M. truncatula* seedlings was measured with a ruler. Roots of *M. truncatula* and *U. dioica* were then cleared in 10% KOH at 80°C for 20 min, rinsed with distilled water and stained with an ink-vinegar solution (5% v:v vinegar with 5% v:v Parker® blue ink; Vierheilig et al. 1998) at 80°C for 5 minutes. For the *H. brasiliensis* plantlets, roots were cleared in 10% KOH at 80°C for 4 hours, rinsed with distilled water, bleached with 3% H_2O_2 at 80°C for 3 minutes, and stained with the ink-vinegar solution at 80°C for 5 minutes.

Roots were examined for AMF colonization under a compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) at x125 magnification. The percentage of total root colonization and percentages of arbuscules and intraradical spores/vesicles were estimated using the magnified intersects method (McGonigle et al. 1990). For each plant, a minimum of 150 root intersections were analyzed.

Enzyme histochemical staining of the AMF mycelium

The mycelium of *R. irregularis* in the HC of each system was extracted following medium solubilization with a sodium citrate buffer (Doner and Bécard 1991). The hyphae were thereafter rinsed to remove the buffer and then stained for detection of succinate dehydrogenase (EC 1.3.99.1) -SDH, alkaline phosphatase (EC 3.1.3.1) -ALP and acid phosphatase (EC 3.1.3.2) -ACP enzymatic activities.

For the SDH activity, the samples were incubated overnight in the staining solution (0.25 M sodium succinate, 0.05 M Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$ and 1 $mg.ml^{-1}$ Nitrotetrazolium blue chloride; Saito 1995) at room temperature in the dark (Zocco et al. 2011). For the phosphatase activities, a

solution of 0.1 M Tris-HCl buffer (pH 8.5) for ALP or 0.1 M sodium acetate buffer (pH 5.5) for ACP was mixed with 1.1 $mg.ml^{-1}$of alpha-Naphthyl phosphate Na salt (Sigma-Aldrich, Steinheim, Germany) and 1.0 $mg.ml^{-1}$ fast blue RR salt (Sigma-Aldrich, Steinheim, Germany). The samples were incubated for 90 min at room temperature in the dark (Zocco et al. 2011).

After the enzymatic staining, samples for SDH, ALP or ACP activities were washed with deionized water for 15 min, counterstained with acid fuchsin (0.1% w/v acid fuchsin/lactic acid) for 15 min, and finally transferred to lactoglycerol (1:2:1 v:v:v lactic acid:glycerol:water) for de-staining and storage (Zocco et al. 2011). For the microscopic observations, the samples were mounted on microscope slides with lactoglycerol. Formazan (for SDH) or Fast blue RR granular precipitation (for ALP and ACP) was assessed with a compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) at x500 magnification. For each sample, at least 150 hyphal intersections were observed following the magnified intersects method (McGonigle et al. 1990). Intersections of hyphae were classified as active or non-active according to the presence or absence of granular precipitates, and the percentage of SDH, ACP and ALP active hyphae was determined.

Statistical analyses

Data analysis was performed with the statistical software Statistica® for Windows (StatSoft 2001). For the percentage of SDH, ACP and ALP active hyphae, the percentage of total colonization, and the percentages of arbuscules and intraradical spores/vesicles, the values were arcsine $\sqrt{(x/100)}$ transformed before statistical analysis. ANOVA analyses were conducted after the verification of the normal distribution, homogeneity of the variances and residuals' independency of the data.

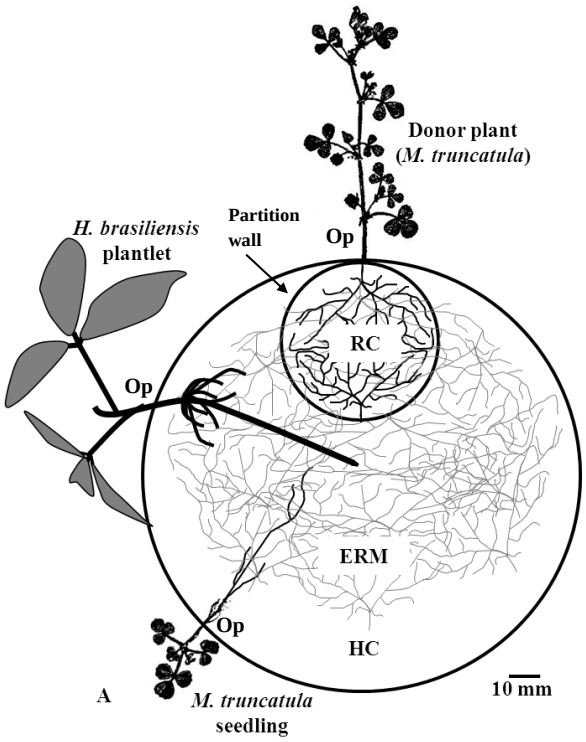


Figure 27. **A.** Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) to study the impact of *Hevea brasiliensis* Müll. Arg. on the mycorrhization of *Medicago truncatula* by *Rhizophagus irregularis* MUCL 41833. Schematic representation of the system showing the disposition of the plants, the root compartment (RC) with the openings (Op) of approx. 2 mm diam. made to allow the development of the plant shoots outside the Petri plate, the partition wall (separation between the compartments), and hyphal compartment (HC), with the extraradical mycelium network (ERM) extending from the RC.

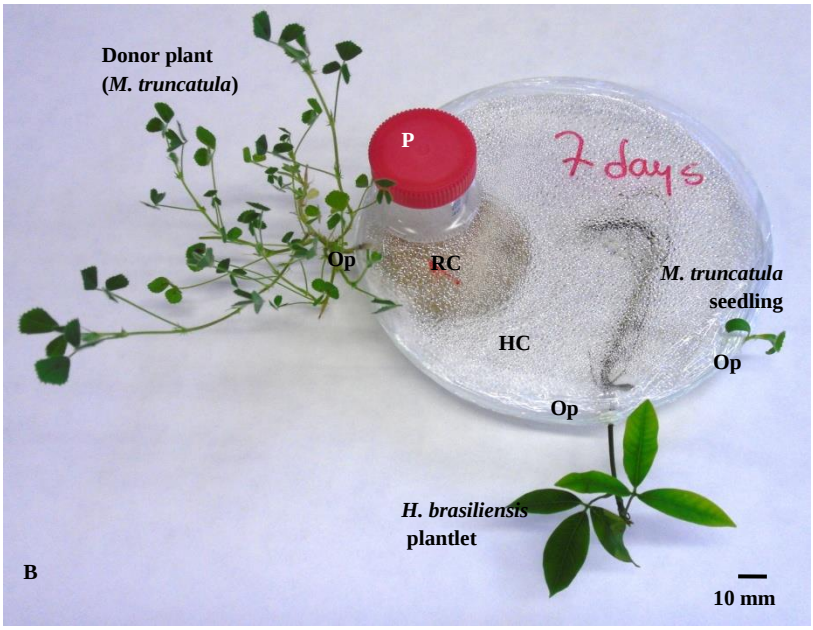


Figure 27. **B.** Mycelium Donor Plant (MDP) *in vitro* culture system adapted from Voets et al. (2009) to study the impact of *Hevea brasiliensis* Müll. Arg. on the mycorrhization of *Medicago truncatula* by *Rhizophagus irregularis* MUCL 41833.
Hevea brasiliensis treatment (HB) system showing the position of the screw cap tube (P) on the system lid, the *M. truncatula* donor plant, and the *H. brasiliensis* test plant.

Results

Impact of Urtica dioica *or* Hevea brasiliensis *on the root colonization of* Medicago truncatula

The presence of *U. dioica* or *H. brasiliensis* negatively impacted the root colonization of *M. truncatula* (Table 7 and 8). In presence of *U. dioica*, the percentages of total colonization and arbuscules were significantly reduced in the *M. truncatula* seedlings as compared to the controls (CT-UD). The percentage of intraradical spores/vesicles colonization was also lower, but the difference was not significant. In presence of *H. brasiliensis* both, the percentages of arbuscules and intraradical spores/vesicles colonization were significantly reduced as compared to the controls (CT-HB). The total root colonization was also lower in presence of *H. brasiliensis*, but the difference was not significant. At the end of the experiments (day 12), the total root colonization was reduced by 30.1% in presence of *H. brasiliensis* and by

29.1% in presence of *U. dioica* as compared with the CT-HB or CT-UD treatments, respectively. In addition, *M. truncatula* roots contained 46.3% and 19.3% less arbuscules in presence of *H. brasiliensis* and *U. dioica* respectively, as compared to the plantlets in the CT-HB or CT-UD treatments (Tables 7 and 8). Independently of the treatment (UD and CT-UD), the first intraradical hyphae were present after 4 days of contact with the mycelium network, and the first arbuscules and intraradical spores/vesicles appeared after 8 days (Table 7). The total colonization and percentages of arbuscules and intraradical spores/vesicles significantly increased with time in presence or absence of *U. dioica* (Table 7).

Neither hyphopodium at the root surface nor root colonization were noticed in *H. brasiliensis* and *U. dioica*. Nonetheless, for *H. brasiliensis,* some hyphae were observed growing along the roots. The total root lengths of *M. truncatula* seedlings were 62.8±9.8 cm and 38.4±5.6 cm in presence of *U. dioica* (UD) and *H. brasiliensis* (HB), respectively and were not significantly different when the seedlings were grown alone (CT-UD or CT-HB treatments; One Way ANOVA, $P \leq 0.05$).

Impact of Urtica dioica *or* Hevea brasiliensis *on the enzymatic activities of* Rhizophagus irregularis

In presence of *U. dioica* seedlings (UD treatment) and *H. brasiliensis* plantlets (HB treatment), the succinate dehydrogenase (SDH) enzymatic activity of the extraradical mycelium growing into the HC (Table 9 and 10) was significantly lower as compared to the controls. A reduction of 23.6% in the proportion of SDH active hyphae in presence of *U. dioica*, and of 15.6% in presence of *H. brasiliensis* as compared with the control treatments (CT-UD or CT-HB) after 12 days of contact with the ERM network were observed. Observations of *M. truncatula* enzymatic activities at 4 and 8 days in the *U. dioica* experiment (Table 9), showed that the proportion of SDH-active hyphae decreased significantly with time in both UD and control (CT-UD) treatments.

Phosphatase activity of the mycelium was only partially affected. Mycelium growing next to the stinging nettle roots had an ALP activity that was significantly lower in comparison with the control (CT-UD; Table 9), but no significant difference was found between the UD and CT-UD treatments for the proportion of ACP active hyphae. Neither the alkaline (ALP) nor the acid (ACP) phosphatase enzymatic activities were significantly influenced by the presence of *H. brasiliensis* (Table 10).

Table 7. Percentages of root colonization of *Medicago truncatula* seedlings after 4, 8 and 12 days of *in vitro* growth in contact with a extraradical mycelium of a *Rhizophagus irregularis* MUCL 41833, in the presence (UD) or in the absence (CT-UD) of *Urtica dioica*

U. dioica	Time (days)	Total root colonization (%)	Arbuscules (%)	Intraradical spores/ vesicles (%)
Present (UD)	4	4.2* ± 0.5	0	0
	8	11.4* ± 1.3	3.1* ± 0.6	2.1 ± 0.7
	12	20.7* ± 0.6	19.6* ± 1.4	11.6 ± 0.2
Absent (CT-UD)	4	6.5 ± 0.9	0	0
	8	18.4 ± 1.3	5.7 ± 0.7	3.1 ± 0.5
	12	29.2 ± 1.2	24.3 ± 1.4	14.2 ± 2.2
Significance of the treatment (P value obtained by the analysis of variance; two-way ANOVA; $P \leq 0.05$)				
UD		<0.001	<0.05	NS
Time		<0.001	<0.001	<0.001
UD x Time		NS	NS	NS

NS not significant. *Values significantly lower than the control (CT-UD). Data represent means +/- SE of four replicates

Table 8. Percentages of root colonization of *Medicago truncatula* seedlings after 12 days of *in vitro* growth in contact with a extraradical mycelium of *Rhizophagus irregularis* MUCL 41833, in the presence (HB) or in the absence (CT-HB) of *Hevea brasiliensis*

H. brasiliensis	Total root colonization (%)	Arbuscules (%)	Intraradical spores/ vesicles (%)
Present (HB)	15.3a ± 1.2	5.1a ± 0.8	2.0a ± 0.3
Absent (CT-HB)	21.9a ± 4.1	9.5b ± 1.4	3.3b ± 0.3

Data represent means +/- SE of four replicates. Significant differences are identified with a different letter for each parameter (one-way ANOVA; $P \leq 0.05$)

Table 9. Percentages of succinate dehydrogenase (SDH), acid phosphatase (ACP) and alkaline phosphatase (ALP) active hyphae of *Rhizophagus irregularis* MUCL 41833 after 4, 8 and 12 days of *in vitro* growth in the presence (UD) or in the absence (CT-UD) of *Urtica dioica*

U. dioica	Time (days)	SDH activity (%)	ACP activity (%)	ALP activity (%)
Present	4	60.3* ± 2.8	29.5 ± 4.9	66.0* ± 1.6
	8	58.5* ± 3.1	28.5 ± 3.5	65.5* ± 3.5
	12	44.5* ± 5.0	30.2 ± 7.6	59.1* ± 0.9
Absent (CT-UD)	4	71.3 ± 4.3	34.5 ± 2.7	73.2 ± 2.0
	8	64.9 ± 6.2	30.1 ± 2.0	71.4 ± 2.7
	12	58.3 ± 2.1	29.2 ± 3.4	68.1 ± 2.8
Significance of the treatment (P value obtained by the analysis of variance; two-way ANOVA; $P \leq 0.05$)				
UD		<0.05	NS	<0.05
Time		<0.05	NS	NS
UD x Time		NS	NS	NS

NS not significant. *Values significantly lower than the control (CT-UD). Data represent means +/- SE of four replicates

Table 10. Percentages of succinate dehydrogenase (SDH), acid phosphatase (ACP) and alkaline phosphatase (ALP) active hyphae of *Rhizophagus irregularis* MUCL 41833 after 12 days of *in vitro* growth in the presence (HB) or in the absence (CT-HB) of *Hevea brasiliensis*

H. brasiliensis	SDH activity (%)	ACP activity (%)	ALP activity (%)
Present (HB)	67.4a ± 1.7	36.4a ± 2.7	64.4a ± 1.7
Absent (CT-HB)	74.9b ± 2.0	37.0a ± 2.0	56.4a ± 2.0

Data represent means +/- SE of four replicates. Significant differences are identified with a different letter for each parameter (one-way ANOVA; $P \leq 0.05$)

Discussion

The establishment of a functional symbiosis between an AMF and the roots of a host plant involves a sequence of events leading to the morphological and physiological integration of the two symbionts (Bonfante and Genre 2008). In plants, colonization may be impaired by a number of factors among which the production of inhibitory molecules (Parniske 2008). Here, we observed that the root colonization of *M. truncatula* seedlings grown in the vicinity of *H. brasiliensis* or *U. dioica* was lower than in the control plants, as well as the metabolic activity of the extraradical mycelium network of the AMF. Similarly, no root colonization was observed in *H. brasiliensis* and *U. dioica* within the time-frame of the experiments (i.e. 12 days). These results suggested that molecules with potential antifungal properties (e.g. UDA and hevein), may be exuded, and thus could limit the AMF colonization of *M. truncatula* roots. The presence of such molecules may further explain the absence or the delay in the colonization previously reported for *U. dioica* and *H. brasiliensis* roots, respectively.

The exudation of inhibitory molecules by *H. brasiliensis* was indirectly detected by growing highly mycotrophic plants in close vicinity of its roots, as was previously reported for *U. dioica* by Vierheilig et al. (1996) and Fontenla et al. (1999). These authors tested the effect of stinging nettle on the root colonization of *Glycine max* and *Pisum sativum* grown in compartmented pots, and observed reduced root colonization of both plants in presence of the stinging nettle.

M. truncatula is a highly mycotrophic plant which colonization in the MDP *in vitro* culture system starts within 2-3 days and reaches a plateau after 12-15 days (Voets et al. 2009). In our experiment, the *M. truncatula* seedlings grown in the presence as well as in the absence of *U. dioica* or *H. brasiliensis* were colonized by *R. irregularis*. Typical intraradical structures (arbuscules, intraradical spores/vesicles and hyphae) were observed. However, the total root colonization of *M. truncatula* was significantly lower in the presence of *U. dioica* than in the control plants, and a significant lower proportion of arbuscules was observed in the presence *U. dioica* and *H. brasiliensis*, suggesting an indirect effect on the intraradical phase of the symbiosis. Other studies conducted with sugar maple seedlings (Barto et al. 2011) and tomato (Roberts and Anderson 2001) grown in the vicinity of *Alliaria petiolata*, another non-host plant, reported a significant reduction of the root colonization and the arbuscule formation. Such inhibitory effects were also associated to the exudation of antifungal compounds produced by *A. petiolata*.

In our experiments, the low percentages of root colonization and the reduced formation of arbuscules may be associated with the reduction in the enzymatic activity observed in the ERM in presence of both stinging nettle and rubber tree plantlets. Arbuscules are specialized structures involved in nutrient exchange between AMF and plant cells (Parniske 2008). Their formation is considered as a worthy indicator of an active mycorrhizal symbiosis (Brundrett 2004; Bonfante and Genre 2008). The lower percentages of arbuscular colonization in *M. truncatula* roots observed in the presence of *H. brasiliensis* and *U. dioica* could be a consequence of the impairment of the metabolic activity of the ERM network. Our results suggested that the exudates produced by these plants may indirectly impact the nutrient exchange within the roots of the receiver *M. truncatula* plant via a reduction of viability and phosphorus metabolism in the ERM network. The lower values of SDH activity observed in the presence of *U. dioica* and *H. brasiliensis* indicated a reduced proportion of viable hyphae in the HC. Furthermore, the reduced activity of ALP in the hyphae of the AMF indicated that the tested plants significantly affected the metabolism of P in the fungal hyphae.

During the 12 days of growth into the MDP *in vitro* culture systems, neither *U. dioica* nor *H. brasiliensis* were colonized, supporting previous observations (Sosa-Rodriguez et al. 2013a; Vierheilig et al. 1996; Fontenla et al. 1999). The MDP *in vitro* culture system was developed for the fast and homogenous colonization of seedlings or micropropagated plantlets (Voets et al. 2009). This system was successfully applied to barrel medic seedlings (Voets et al. 2009), but also to potato (Gallou et al. 2010), banana (Koffi et al. 2012) and rubber plantlets (Sosa-Rodriguez et al. 2013a). While the three first plant species reached heavy root colonization values within the *in vitro* period of sub-cultivation (~3-4 weeks), *H. brasiliensis* root colonization markedly exceeded this duration (~7-8 weeks).

Thus, the observed absence of roots colonization in *U. dioica* by the active ERM network of *R. irregularis* was a confirmation of its status of non-host plant. For *H. brasiliensis*, the results suggested the existence defense mechanisms via the exudation of inhibitory molecules that may impair early root colonization by the AMF. However, this inhibition seems to be transient, as *H. brasiliensis* could be colonized after 7 weeks of contact with an ERM network under similar experimental conditions (Sosa-Rodriguez et al. 2013a).

The exudation of inhibitory compounds was probably linked to a defense strategy against fungal pathogens (hevein was indeed reported to reduce the

growth of several fungi; Van Parijs et al. 1991), but could also a defense response elicited in the young *H. brasiliensis* trees by the AMF itself (Kloppholz et al. 2011). The transient non-host plant behavior displayed by the *H. brasiliensis* plantlets towards AMF would thus be a collateral effect of a defense strategy developed by the tree to facilitate its establishment. Kloppholz et al. (2011) found that if AMF and plants have a long history of mutual association, during an early phase of the symbiosis the plant can react to the colonization by the AMF, inducing transient defense and stress responses. It was therefore suggested that AMF, to establish long-term biotrophic relationships with their hosts, must either actively suppress or avoid these plant defense responses.

Schwob et al. (2000) already observed a transitory enhancement of some enzymes involved in the lignin metabolism in roots of *H. brasiliensis* seedlings inoculated with *F. mosseae.* They hypothesized that the AMF elicited a temporary defense on the natural rubber roots. Whatever the mechanism involved, our hypothesis that *H. brasiliensis* roots produce and exude molecules with antifungal properties is therefore plausible. The antifungal capacity of hevein, a protein similar to UDA present in the latex of *H. brasiliensis* has already been established (Van Parijs et al. 1991; Martin 1991; Kanokwiroon et al. 2008).

However, clarification of the involvement of hevein would need to be performed as its exudation by *H. brasiliensis* roots was never documented. The detection of UDA, hevein (or other antifungal compounds) exuded during the growth of the *M. truncatula* seedlings in the presence of *U. dioica* or *H. brasiliensis* could also be quantified. The dynamic of root colonization could be paralleled with the detection of the specific compounds exuded in the culture medium during the time course of the experiment. In this case, specific protein detection techniques as Western blot could be used (using the suitable antibody; Laukkanen et al. 2003). It is also worth to notice that the compounds exuded by *H. brasiliensis* could be either less active against *R. irregularis* than UDA, or exuded in lesser amounts than by *U. dioica,* as the ERM was able to grown close to *H. brasiliensis* roots during the first 12 days of contact (this behavior was never observed with *U. dioica*), and the P metabolism (i.e. the ACP and ALP enzymatic activities) of the fungus was not significantly reduced in the presence of the rubber plantlets.

As a conclusion, we demonstrated that the highly mycotrophic plant *M. truncatula* presented lower colonization in the presence of *H. brasiliensis* or *U. dioica* than control plants grown alone. This result, along with the lower enzymatic activity noticed in the ERM network of the AMF, suggested the

exudation of diffusible molecules from the roots of *H. brasiliensis* as well as *U. dioica* having antifungal activity, and thus leading to a lower colonization of *M. truncatula*. The exudation of these antifungal molecules by the roots of *H. brasiliensis* and *U. dioica*, could also explain the delay or the absence of root colonization in these plants, respectively. However, the physiological mechanisms and ecological reasons that would explain the transient or non-host behavior of these plants need to be further explored.

Acknowledgements

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General discussion

Vegetative propagation by budding is the current technique applied in the commercial plantations for breeding and propagation of *H. brasiliensis*. In the recent decades, alternative propagation techniques such as the *in vitro* culture of *H. brasiliensis* by somatic embryogenesis or by microcuttings have been developed to produce self-rooted and rejuvenated plantlets, by-passing some of the limitations of the conventional budding, e.g. the incompatibility between the stock and the scion, or premature aging of the grafted material (Cardinal et al. 2007; Carron et al. 2009).

Integration of micropropagation techniques in *H. brasiliensis* breeding is a promissory research field that has brought successful results, in spite of the fact that *H. brasiliensis* is a difficult species for *in vitro* culture (Montoro et al. 2012). Nowadays, i*n vitro* produced *H. brasiliensis* clones are mostly used for research purposes, especially in genetic engineering and mass propagation studies (Leclerck et al. et al. 2012; Montoro et al. 2012). This selected plant material also represents an interesting tool for specific studies under controlled conditions, such as the *in vitro* association with AMF (see Declerck et al. 2005 for a review).

Furthermore, it has been observed that *in vitro* mycorrhized plantlets can be better protected against eventual stresses of the acclimatization if they are associated with beneficial soil microorganisms during the *in vitro* growth (Liu and Yang 2008). AMF have been successfully applied during the first steps of the *ex vitro* acclimatization, reducing the duration of the hardening phase and stimulating the plant growth (Hazarika and Bora 2006; Kapoor et al. 2008).

The *in vitro* association with AMF under autotrophic culture conditions (e.g. in the MDP *in vitro* culture system) has made possible the utilization of AMF as active inoculum for the mycorrhization of plantlets under *in vitro* culture conditions. However, the association of AMF to a whole plant *in vitro* remains a challenging process, especially because the AMF and the host plantlet are dissimilar organisms, and may have different growth rates and/or nutrient requirements (Declerck et al. 2005; Desjardins et al. 2005).

During this thesis, it was necessary to adjust different *in vitro* culture conditions to obtain the first *H. brasiliensis* plantlets successfully mycorrhized with AMF in autotrophy (**Chapter 1)**. There are no previous

published works on this matter, and the potential of this technique is huge. We selected suitable culture conditions for the early mycorrhization of the rubber tree. The results of our work may be used as the starting point in novel research about the biology and the genetics of rubber plants produced *in vitro*, when exposed to challenging environmental conditions (e.g. drought stress or pathogens attack).

AMF are capable to stimulate the plant defence, and the presence of these fungi within the root before the contact with the pathogen seems to be important to favor the plant protection (Gallou et al. 2011). Thus, the system developed during our research could be used (and/or ameliorated) in future experiments of interaction between *H. brasiliensis*, AMF and pathogens of the rubber plantations (e.g. the nematode *M. exigua*, or the fungi *M. ulei*, *R. lignosus* and *P. noxius*). Indeed, the MDP *in vitro* culture systems allow the establishment of experiments under controlled conditions that assure reduced variability and allow the incorporation of specific techniques such as molecular biology or biochemistry (Declerck et al. 2005).

Besides, the capacity of AMF to improve the growth of rubber plants could be exploited in the nursery, to stimulate the vigour and the rapid growth recovery of the micropropagated rubber material. Thus, the integration of AMF to the mass propagation of natural rubber *in vitro* plantlets could be achieved in a near future, when the mass micropropagation techniques will be enough developed to be applied on a commercial scale (in fact, AMF are considered as one of the tools that may be used to achieve this goal).

During our experiments, the colonization of the rubber roots was exceptionally slow (approx. 7 weeks), compared to the time necessary to obtain intraradical root colonization of other plants using similar conditions (see Gallou et al. 2011 or Koffi et al. 2012 for some examples). We observed that the early root production of rubber plantlets clone PB 260 and their consequent mycorrhization by *R. irregularis* MUCL 41833 appeared to be highly controlled by the plant, making difficult the amelioration of the root growth/production and of the early root mycorrhization by using a traditional way (i.e. changing basic culture conditions as the presence of CO_2 in the growth chamber, the medium pH, or the activated charcoal -AC content).

New specific strategies are mandatory (see **Perspectives**). Nevertheless, the results obtained during this thesis are a valuable "first approach" to the biology of the symbiosis of this couple when working *in vitro*. Thus, taking into account the main outcomes of this research (but also the main

unresolved questions), a model for the *in vitro* mycorrhization of *H. brasiliensis* clone PB 260 by the fungus *R. irregularis* MUCL 41833 was proposed at the end of this section.

We evaluated root development and colonization of rubber plantlets under an increased CO_2 atmospheric concentration, combined with the addition of MES buffer to the growth medium, and pre-treatment of the plantlets with IBA **(Chapter 2).** The best conditions to obtain larger roots and higher colonization values than in control plantlets were exposition to elevated CO_2 and growth in the presence of MES. A similar response to the CO_2 treatment of *in vitro* plantlets is well documented (Douds Jr 1997; Pawlowska et al. 1999; Karandashov et al. 2000), and the reason is also known: CO_2 stimulates photosynthesis of *in vitro* grown plantlets (Liu and Yang 2008) by increasing the carbon allocation to the roots, which show better growth and improved mycorrhization (Hodge 1996; Treseder and Allen, 2000).

In contrast, the reason for the enhanced AMF growth in presence of MES remains speculative. Vilariño et al. (1997) and Bago et al. (2004) suggested that the MES buffer may serve as sulfur source to the fungus, stimulating its growth. It could also be hypothesized that buffering the pH with MES may prevent the acidification of the culture medium that could impair the AMF growth and/or the root colonization. It is known that the exudation of organic acids and protons by the roots may change the pH of the substrate (Neumann and Römheld 1999; Dakora and Phillips 2002). van Aarle et al. (2002a) observed that lowering the pH of the substrate impaired the colonization of *Plantago lanceolata* by the AMF *G. intraradices* and *Scutellospora calospora*. These authors argued that pH low values caused a stress response of the fungi affecting its colonization capacity. This kind of response could be regulated by the addition of MES. In addition, regulation of the pH can facilitate the solubilization and further uptake of the nutrients by the plantlets and by the fungus (Medeiros et al. 1993; Dakora and Phillips 2002; George et al. 2008).

We also exposed the *H. brasiliensis* plantlets to 10 μM IBA added to the mixotrophic culture medium for one week, and then transferred the plantlets to the MSR medium. This treatment did not stimulate the root elongation and seemed to block the mycorrhization (**Chapter 2**). We only tested one IBA treatment that was probably too long (and/or too concentrated) to obtain a positive effect on the rubber roots and on their mycorrhization. It is known that prolonged exposition to IBA can have inhibitory effects on the rooting process. Moreover, IBA treatment can be more effective for the rooting of micropropagated plantlets obtained by

microcuttings, instead of plantlets produced by somatic embryogenesis (de Klerk et al. 1999; Montoro, *personal communication*). In addition, it has been observed that IBA can be inhibitory for AMF development (see Gryndler et al. 1998; and this work).

Whatever the factor assayed during this thesis, root colonization was observed after a period of approximately 7 weeks of *in vitro* culture of natural rubber in the extraradical mycelium (ERM) network of *R. irregularis*. When the plantlets were inoculated with spores, the first traces of mycorrhization were observed after 15 to 20 weeks (**Chapter 1**). We also tested two different periods of contact between the *H. brasiliensis* plantlets and the ERM network of *R. irregularis* (**Chapter 2**). The first group of plantlets (see exp. 1) grew inside of a young ERM mycelium network. That means, during the first weeks of autotrophic growth, the mycelium of *R. irregularis* was not completely spread in the hyphal compartment -HC. Thus, the first roots of *H. brasiliensis* produced in autotrophy were not in contact with a large biomass of AMF. In addition, the contact between the fungus and the roots lasted only 5 weeks.

In contrast, for the second group of plantlets (see exp. 2; **Chapter 2**), the new roots produced by *H. brasiliensis* were immediately in contact with the mycelium of the fungus. Also, the roots grew inside the ERM network during 13 weeks. Against the most optimist predictions, a fully developed ERM and a longer exposition to the AMF did not accelerate the onset of root colonization. In addition, root colonization with production of arbuscules was mostly restricted to newly produced roots. As we firmly thought that the presence of an active and well developed mycelium network could favour the early colonization of the roots, as demonstrated earlier by Voets et al. (2009). The condition of mycorrhization on a pre-established mycelium network of the AMF in the HC was maintained along the subsequent experiments (**Chapters 3 and 4**).

The apparent "absence of reaction" of the rubber roots to the presence of the AMF was unexpected, since *H. brasiliensis* is a mycotrophic plant which associates with AMF, producing important colonization values, even in heterogeneous conditions of fertility or drowning (see **State of the art** Section and Feldmann 1994). Thus, the observed initial behavior indicated that somehow, the dialogue between *H. brasiliensis* and *R. irregularis in vitro*, which starts before the physical contact of the roots and the mycelium (Bonfante and Genre 2010), did not happen in the same way than for other mycotrophic plants (see "Early interaction between plants and AMF" part of the **State of the art Section** for details).

We observed that the hyphae of *R. irregularis* did grow next to the rubber roots, extending along the roots and branching during the first two weeks of contact (**Chapter 4**). This result indicated that as similar to other mycotrophic plants, *H. brasiliensis* would be capable to produce root exudates, containing attractive compounds (i.e. strigolactones) to indicate their presence to the AMF (Akiyama et al. 2005). However, the formation of hyphopodia on the surface of the roots was not observed at the same time, and the active colonization (i.e. the formation of arbuscules) only started some weeks later (after about 9 weeks of growth inside the ERM). Several questions arise here: Is the young rubber plant capable to block the entering of *R. irregularis* to the roots? If yes, Why? And more importantly: How? What is blocking/retarding the advancement of the root colonization?

It is probable that the recognition process between *H. brasiliensis* starts as is generally described for the AMF symbiosis. Thus, in addition to attractive compounds produced by the plant, AMF presymbiotic mycelium also produces specific diffusible molecules (i.e. the Myc factors) that are perceived by the host plant before the physical contact with the fungus. The plant responses to Myc factors are diverse (ranging from the molecular to the organ level), and the whole process has not been fully described. What is already known is that these factors (and/or other signaling molecules) could be recognized by specific receptors proteins in the plant membrane (see Bonfante and Genre 2010 and "Early interaction between plants and AMF" part of the **State of the art** Section for details).

It is also accepted that different signaling pathways can induce the expression of different plant genes involved in the early plant response to AMF (Gallou 2011). More importantly, not all of the signaling pathways would be positively triggering the root colonization. For instance, different signaling pathways regulating the plant defence response during AMF colonization have been identified (Pozo and Azcon-Aguilar 2007). The transiently increased expression of plant defence genes during the early stages of root colonization, followed by a decline in their expression has been also reported (See Gallou 2011 for details). Current data suggest that the host plant initially treat symbiotic fungi as potential invaders and activates a defence program, which is then countered by the mycorrhizal symbiont (Zamioudis and Pieterse 2011).

Thus, a potential host plant could have and unexpected "protective reaction" against the colonization. In fact, this behavior has been well documented (Vierheilig and Bago 2005; Stinson et al. 2006; Yun and Choi 2002 Barto et al. 2011), and fortunately (or unfortunately?), numerous

inorganic or organic compounds such as flavonoids, jasmonic acid, phytoalexins, H_2O_2 metabolites, phenols, chitinases… have been identified. Therefore, a host plant is capable to exudate compounds that can "down regulate" the growth of AMF, acting on different stages of development of the symbiosis from spore germination, to root colonization and production of extraradical mycelium (Vierheilig 2004).

We assumed that young *H. brasiliensis* plantlets could be triggering some kind of signaling pathway to induce the production of root exudates capable to act against *R. irregularis*. To understand why, it may be useful to evoke the behavior of the plants that naturally grow without associating with AMF (called non mycorrhizal, non mycotrophic, or non-host plants -NM). The roots of NM plants are unable to support a true colonization with the development of functional arbuscules. Furthermore, these plants developed different strategies to avoid the growth of AMF in their roots, as the production and exudation of inhibitory or antifungal compounds (Vierheilig and Bago 2005; Smith and Read 2008).

It is true that *H. brasiliensis* is not a non-host plant. After an initial delay, root colonization starts and develops inside the roots. However, the classification of the plants as mycorrhizal or non mycorrhizal is not black and white. It has been established that mycorrhizal plants are in a continuum, where different values of dependency to the association can be found, ranging from the obligate mycorrhizal to the non-mycotrophic plants. Moreover, the dependency of a plant to symbiosis could be influenced by different plant and environmental factors (Brundrett 2002; Janos 1980; 2007). Thus, we could hypothesize that the natural rubber plantlets (and maybe also the seedlings) act as a non-host during the first days of their development, shifting to a more "mycotrophic state" later, when the plant is more developed and able to support the root colonization.

Thus, the transient non-host behavior displayed by the rubber plantlets would be an effect of a defense strategy developed by the young tree to facilitate its early establishment (see Kloppholz et al. 2011 and Chapter 4 for details). It has to be recalled that the association with AMF always implicates a carbon cost for the host plant (Johnson et al. 1997; Vannette and Hunter 2011). Thus, the plantlets could be interested in retarding the arrival of the fungus to the roots during their early growth and establishment, when their own organs are the primary sink for carbon allocation. The temporary activation of specific defence mechanisms in the roots of rubber seedlings in the presence of the AMF *Glomus mosseae* has been already described (Schwob et al. 2000).

The identification of the compounds that could be retarding the advancement of the root colonization in the roots of the *H. brasiliensis* plantlets was out of the scope of this work, and remains to be determined (see **Perspectives**). Nevertheless, we measured the impact of the presence of *H. brasiliensis* on the *in vitro* colonization of *M. truncatula,* a highly mycotrophic plant, in order to find an indirect evidence of the root exudation of anti-AMF substances by *H. brasiliensis* (**Chapter 4**).

We used the non-host plant *U. dioica* as a positive control, since its ability to exudate antifungal compounds (e.g. UDA) is already known. We expected to observe a detrimental effect on the root colonization of *M. truncatula* in the presence of *U. dioica* that we could contrast with a similar reaction in the presence of *H. brasiliensis* plantlets (see "Early interaction between plants and AMF" part of the **State of the art Section** and **Chapter 4** for details). We observed that root colonization of *M. truncatula* grown in the vicinity of *H. brasiliensis* or *U. dioica* was decreased. Besides, the metabolic activity of the extraradical mycelium network of the AMF was also impacted in the presence of both plants. These results supported the exudation of antagonistic molecules by the roots of *H. brasiliensis*.

It could be possible that the inhibitory compounds present in the exudates of *H. brasiliensis* plantlets contains latex and/or latex related proteins, like the hevein-like AMPs (AntiMicrobial Peptides), which are part of the innate immunity of the plants, establishing a first line of defence against pathogens. All plant organs express AMPs constitutively or in response to microbial challenges (see Stotz et al. 2013 or Odintsova and Egorov 2012 for a comprehensive review). In addition, the latex cells can be found in the taproot and in the lateral roots of *H. brasiliensis* seedlings and *in vitro* produced plantlets (Schwob et al. 1999; Putranto et al. 2012).

Hevein-like AMPs have structural similarity with hevein, a small protein with antifungal properties that is a major component of the latex of *H. brasiliensis* (Gidrol et al. 1994; Van Parijs et al. 1991). Mature hevein contains a conserved chitin-binding domain, which is also present in all the hevein-like AMPs. In addition to hevein, other members of this family are lectins (as UDA), chitinases, some wound-induced proteins, and a number of peptides later classified as "hevein type" AMPs (Odintsova and Egorov 2012; Stotz et al. 2013).

The antifungal activity of the hevein-like AMPs has been reported against both filamentous fungi and yeast. Furthermore, some of these chitin-binding proteins have shown antibacterial and insecticidal activity (see Odintsova

and Egorov 2012 for a review). However, the mode of action of this family of compounds is poorly understood. It has been suggested that the capacity to bind chitin could interferes with the correct assemblage of the fungal wall (Broekaert et al. 1989; Van Parijs et al. 1991). It has also been proposed that the degree of blockage on the mycorrhization could be dependent of the amount of inhibitory substances that reaches and accumulates in the substrate (Fontenla et al. 1999). Thus, exuded at the right concentrations, antimicrobial peptides synthetized by the rubber plantlets could be harmful to the AMF mycelium.

A second result that may be considered as indirect proof of the exudation of inhibitory compound by *H. brasiliensis* was obtained after the addition of activated charcoal (AC) to the culture medium (**Chapter 3**). AC was added in order to stimulate the early root production and subsequent root colonization of *H. brasiliensis*, as has been reported for other micropropagated plants (Pan and Staden 1998; Thomas 2008). We hypothesized that AC may sequester inhibitory compounds present in the root exudates of *H. brasiliensis* that could accumulate in the culture medium, and affect the growth of roots and/or the AMF. During our experiments, we clearly demonstrated that AC significantly increased the root length and colonization of the rubber tree plantlets. Nevertheless, we did not observe any effect on the onset of the mycorrhization, or on the activation of lateral root meristems, indicating that additional factors (probably controlled by the plant physiology) would be influencing the root development and mycorrhization process.

During the colonization process of a typical mycotrophic plant, the initial exchange of signal molecules is followed by the contact of the AMF hyphae and the surface of the plant root. AMF specifically select the location for start the root penetration. They can extend for several centimeters along the root surface forming long hyphae. Then, they swell, flatten on the cell wall of a few epidermal cells and branch repeatedly to develop the hyphopodium. The exact selection mechanism of the penetration point is unknown, but it has been observed that AMF hyphal branching concentrates in the vicinity of young lateral roots, which are also considered as the primary site of AMF colonization (Bonfante and Genre 2010).

Actually, there is a strong relationship between the root anatomy (and age) and the root colonization. For instance, Guo et al. (2008) observed that lateral young roots are more susceptible to AMF colonization than older roots with secondary development. Besides, young sections of growing roots are more susceptible to AMF penetration, especially in areas where exodermis is

developing, i.e. before complete suberization of the exodermal root cells (Vidal et al. 1992), and/or in zones with presence of specific passage cells (Brundrett 2002; Sharda and Koide 2008).

H. brasiliensis young plants have a hierarchical root morphology, presenting a strong taproot and several lateral roots (see "The root system" part of the **State of the art Section** for details). The taproot has a large apical diameter, well-developed stele and primary and secondary growth. Lateral roots do not have secondary growth (Schwob et al. 1999; Putranto et al. 2012), and appear to be highly specialized for the nutrient uptake (Putranto et al. 2012). Thus, a close interaction of these roots with AMF may be expected.

Thus, a treatment which could stimulate the production of lateral roots in the rubber plants appeared to be appropriate. We combined the addition of AC to the growth medium with the pruning of the taproot of the *H. brasiliensis* plantlets. Both treatments were applied to induce the production of lateral new roots. Nevertheless, in our study, the AC addition and the pruning of the taproot did not induce early root formation. This failure suggested that the emission of lateral roots (i.e. the induction of root meristems that originate new lateral roots) is highly controlled (see the Discussion section of **Chapter 3**), and probably pre-determined by the genetic program of the plant, as previously suggested by Thaler and Pagès (1997). Thus, more elaborated treatments than the modification of some culture conditions (e.g. the addition of AC) should be tested (see Perspectives).

For instance, the generation of transformed clones with accelerated rhizogenesis could be considered. Alternatively, the addition of an *in vitro* "pre-rooting stage" before putting the plantlets in contact with the ERM network of the AMF could be assayed. In that way, the *H. brasiliensis* plants would have enough young roots ready to be colonized, and also, as the plantlets are expected to be older (and then, more developed), the initial "defence response" could be minimized. Unfortunately, this option requires the addition of another *in vitro* stage the duration of the whole micropropagation process. The interest of such a choice is questionable.

To conclude, we expected that the results of this thesis and the model *H. brasiliensis*/AMF *in vitro* proposed below, will open the door for further studies on the factors involved in this intimate association, and for the production of mycorrhized *H. brasiliensis* plantlets with improved growth at transfer to the field.

Model for the early *in vitro* mycorrhization of *H. brasiliensis* clone PB 260 with the AMF *Rhizophagus irregularis* MUCL 41833

1. Transfer of *H. brasiliensis* plantlets from the myxotrophic to the autotrophic culture medium and first days of growth: best culture conditions

The improvement of the culture conditions for the transfer of *H. brasiliensis* plantlets from the myxotrophic to the autotrophic culture medium should include 1000 μmol mol^{-1} of CO_2 in the growth chamber. With this treatment, the photosynthesis, the carbon allocation to the roots, and consequently their growth and mycorrhization may be improved. Furthermore, the addition of 10 mM of MES to the culture medium may enhance the growth of the AMF, probably by acting as a potential source of nutrients (e.g. sulfur) and/or by regulating the pH of the medium, eliminating the acidification of the medium as a source of stress for the fungus and/or facilitating the solubilization and further uptake of the nutrients by the plantlets and by the fungus.

In order to obtain root growth stimulation (elongation or meristems stimulation) and mycorrhization of micropropagated rubber plantlets, different concentrations and treatment conditions with the growth regulator IBA should be tested, avoiding the concentration and/or the methodology tested during this work. Furthermore, IBA treatment should be preferably applied only if working with *H. brasiliensis* microcuttings.

2. Inoculation of AMF in the culture media: using the ERM network

When using *R. irregularis* MUCL 41833 to obtain the *in vitro* mycorrhization of *H. brasiliensis* clone PB 260 plantlets, an ERM network of the fungus should be preferred, instead of using non germinated viable spores. Inside the ERM, root colonization with the formation of arbuscules may be observed after approximately 7 weeks of contact between the roots and the mycelium. As root colonization appears to be mostly restricted to newly produced roots, a fully developed ERM network of this fungus should be used, in order to assure the early contact between the susceptible roots and the fungus.

3. **First days (weeks) of contact between the plantlet and the AMF: What is really happening when "nothing happens", and the *H. brasiliensis* response "*be careful; don't colonize my roots when I'm too young, I can harm you*"**

Hevea brasiliensis is a mycotrophic plant. Its mycorrhization starts by a presymbiotic dialogue with *R. irregularis* that takes place in the absence of physical contact between the roots and the hyphae. The initial recognition would be probably mediated by signal molecules produced by the plantlets and by the fungus. Some of the fungal molecules could trigger a transient defence response in the young rubber plantlets. Then, *H. brasiliensis* could exudate specific quantities of compound(s) with antifungal/anti-AMF activity, in order to retard the penetration of *R. irregularis* into the cortex of the roots, probably by disturbing the correct assemblage of the fungal wall. The objective would be the prevention of the early colonization of its roots (that implicates a carbon cost for host the plant).

Eventually, the plantlet continues to grow, and other signaling pathways would be activated (changing the quantity, and/or the quality of the root exudates) to allow the normal development of the symbiosis. One gram per litre of culture medium of activated charcoal can be added to the culture medium, in order to limit the effect of organic compounds exuded by *H. brasiliensis* on the growth of *R. irregularis*, and to increase the elongation and the colonization of the lateral roots.

4. **Some weeks later: Evolution of the root growth of the rubber plantlets and active colonization and the AMF response "*I only like your young roots*"**

The lateral secondary roots of *H. brasiliensis* clone PB 260 presenting active growth and young, primary tissues are the preferred site for the formation of hyphopodia, the penetration and further colonization by the AMF *R. irregularis* MUCL 41833 when growing together under in vitro autotrophic culture. Thus, the presence of this kind of roots will be mandatory, in order to assure the development of the symbiosis.

Conclusions

The world demand for natural rubber is still increasing and thus necessitates biotechnological innovations such as the propagation of elite planting material, the characterization and conservation of genetic resources and the development of transgenic plants capable to overcome a/biotic stresses, and to produce high quantities of latex. The integration of *in vitro* micropropagation of *H. brasiliensis* with the inoculation of AMF represents a promising approach which may contribute to the achievement of these goals.

Here, we investigated the effect of the culture conditions on the interaction between the AMF *Rhizophagus irregularis* MUCL 41833 and *Hevea brasiliensis* clone PB 260 using a tailor-made *in vitro* cultivation system. The long-term objective of this research is the utilization of the system for the study of the genetics and the physiology of the rubber plantlets during the initial steps of the association with AMF, and also for the production of vigorous *in vitro* mycorrhized plantlets with improved growth at the transfer to the field. Indeed, obtaining in *vitro* root colonization in a time frame compatible with the *in vitro* production cycle of *H. brasiliensis* will be essential for the mass production of *in vitro* mycorrhized plantlets.

Our attention was particularly focused on the culture conditions that could increase the root growth and further colonization by the AMF. Three central questions were addressed:

1. **Is it possible to develop an autotrophic *in vitro* culture system adapted to the morphology of *H. brasiliensis* plantlets? Can we use this system to associate *R. irregularis* MUCL 41833 to *H. brasiliensis* plantlets?**

The mycelium donor plant (MDP) *in vitro* culture system was developed by Voets et al. (2009) for the fast and homogenous mycorrhization of several herbaceous plants (e.g. *M. truncatula*, *S. tuberosum*, *Musa* spp. …) by means of the production of an active extraradical mycelium (ERM) network. Some technical adaptations were made to this system to allow the growth of *H. brasiliensis* (e.g. the size of the system; see **Chapter 1 and 2**).

Rubber is a tree which roots are large and thick (i.e. they have ligneous tissues with secondary growth; Nicole 1984). Thus, the bi-compartmented system developed by Voets et al. (2005 and 2009), and further adapted to the

in vitro mycorrhization of banana plants - which are also large - (Koffi et al. 2009), was adjusted to the rubber tree plantlets, by combining two Petri plates, in order to physically separate the root compartment (145 mm diameter), in which the AMF is associated to a *M. truncatula* plantlet, from the hyphal compartment (55 mm diameter), in which only the mycelium of the AMF is allowed to spread.

The system was effective. Root colonization with development of arbuscules and production of intraradical spores/vesicles was obtained after 7 to 9 weeks of contact between the ERM network and the roots. However, the duration was too long for potential practical applications. One of the possible causes of this delay could be the limited root production of the plantlets during the first weeks of autotrophic culture. This observation oriented our study to the search of culture conditions that could stimulate root production and accelerate their colonization by *R. irregularis*.

2. **Is it possible to improve the root development/production of *H. brasiliensis* and its *in vitro* colonization by modifying the culture conditions (e.g. CO_2, IBA, MES, AC, taproot pruning treatments)?**

The study of the combined effects of the CO_2, IBA and MES treatments (**Chapter 2**) allowed us to determine that: (1) the pretreatment of the rubber plantlets with 10µM of IBA was detrimental to the root colonization and (2) the combination of elevated CO_2 (1000 µmol mol^{-1}) in the growth chamber and addition of 10 mM MES to the culture medium improved the root colonization. Only the last two treatments were used in further experiments.

In **Chapter 3**, we observed that the addition of 1g l^{-1} of AC to the MSR medium (in addition to the 10 mM MES supplement, and the placement of the plantlets under 1000 µmol mol^{-1} of CO_2 in the growth chamber) increased the total root length and colonization. In contrast, the pruning of the taproot did not did not favor the root colonization. Importantly, neither the addition of AC nor the taproot pruning influenced the production of new early secondary roots (ESR) or the earlier colonization of the pre-existing roots. Our results suggested that the presence of newly produced roots was not the only factor necessary to obtain faster *in vitro* root colonization of the rubber tree seedlings. We proposed that the presence of AMF could trigger the exudation of secondary metabolites and/or antifungal molecules, which could transiently affect the colonization, and thus delay the establishment of a functional symbiosis.

3. Do *H. brasiliensis* root exudates have antifungal properties? Do these exudates impact the root colonization of *Medicago truncatula* plants by the AMF *R. irregularis*?

Roots exudates are a co-evolutionary strategy to manage the dialogue between the plants and between the plants and the rhizosphere biota (Bais et al. 2006) that can significantly influence the interaction between the plants and their AMF symbionts. Root exudates may contain antifungal molecules inhibiting or delaying root infection by pathogens, but also colonization by AMF.

We conducted an experiment in which the root colonization of a highly mycotrophic plant (*M. truncatula*) grown in the vicinity of *H. brasiliensis* or *U. dioica* (a non-host plant producing antifungal molecules) was monitored and compared to a control (i.e. in absence of *H. brasiliensis* or *U. dioica*) plantlet. Root colonization, arbuscules production, and the enzymatic activity measured in the ERM network of *R. irregularis* were reduced in presence of both plants, suggesting that the intraradical phase of the AMF and its metabolism were affected. These results indirectly suggested the presence of diffusible molecules exuded from the roots of *H. brasiliensis* (and *U. dioica*) with possible anti-fungal activity.

We can summarize the main results of this thesis:

- The first *in vitro* mycorrhization of *H. brasiliensis* model was established.
- Specific factors that favored the *in vitro* root growth and subsequent mycorrhization of the rubber plantlets were evaluated. Notably, the addition of MES (10 mM) and activated charcoal (1 g l^{-1}) to the MSR medium, coupled to an increase of CO_2 (1000 µmol mol^{-1}) in the growth chamber improved root growth and colonization.
- The root colonization of *H. brasiliensis* plantlets was obtained only after approx. 7 weeks of contact of the roots with the extraradical mycelium of the AMF.
- *H. brasiliensis* plantlets presence significantly decreased the root mycorrhization of neighbouring plantlets of *M. truncatula.* This finding suggested the exudation of substances capable to retard the root colonization by *R. irregularis* in rubber plantlets and/or neighbouring plants.

Perspectives

Notwithstanding the outcomes of this thesis, there are at least two central questions that need to be answered to understand the factors that are regulating the early mycorrhization of the rubber plantlets.

1. Which factors are controlling the root production and subsequent colonization of the *in vitro* produced *H. brasiliensis* plantlets? How could these factors be modified?
2. Will the *in vitro* association of *H. brasiliensis* with AMF improve the performance of the plantlets during their acclimatization in the nursery and successive transplantation to the field?

Increase of root production and subsequent root colonization of *in vitro* produced rubber tree plantlets

To improve the production of roots at the early stages of *in vitro* growth of *H. brasiliensis*, additional treatments reported in the literature to stimulate root growth should be assayed in combination with AMF. Here, we selected the factors expected to have a major influence on the AMF/*H. brasiliensis* association: (1) light, (2) addition of growth regulators to the MSR medium, (3) co-inoculation with other beneficial soil microorganisms, (4) plantlets genetics, root anatomy and age, and (5) root exudates.

Light. Several studies on plant micropropagation under autotrophic culture conditions have combined increased CO_2 in the growth chamber with a high photosynthetic photon flux density - PPFD (Thi Nguyen et al. 2001; Lui and Yang 2008). It is not excluded that *H. brasiliensis* plantlets require specific light conditions to grow, because it is recognized that *in vitro* raised plants are very sensitive to high PPFD. The combination of high CO_2 in the atmosphere, and the variation of the PPFD applied at different times of the *in vitro* mycorrhization (e.g. low density during the first days, and increased PPFD during the next weeks) should be investigated to establish the adequate light-CO_2 combination that may increase the overall growth of the

rubber plantlets and the root production and thus, the root colonization by AMF.

Growth regulators. A different methodology of treatment, and/or new concentrations of IBA should be assayed (see **Chapter 2** and **General discussion**) in order to establish a proper concentration that could induce production of roots without impacting the AMF.

Alternatively, other plant regulators with confirmed effect on the of root production, growth and mycorrhization such as 6-benzyladenine; a-naphthalene acetic acid or abscisic acid -ABA or gibberellic acid (Normah et al. 1995; Gryndler et al. 1998; Foo et al. 2013) could be tested on the rubber plantlets, by addition to the culture medium before or during the association with the ERM network of *R. irregularis* (see also General discussion). Mutant plantlets deficient in selected plant hormones could be used to determine if the colonization of the roots is altered by the level of these hormones or similar signaling compounds. Also, the expression of genes activated during mycorrhizal colonization could be monitored (Foo et al. 2013).

For instance, Herrera-Medina et al. (2007) studied the mycorrhization of tomato mutants with reduced ABA production, finding a lower susceptibility of the mutant plants to the root colonization, in comparison with the wild plants. A similar experiment using transformed *H. brasiliensis* plantlets with impaired ABA production could be developed. Alternatively, the exposition of normal rubber plantlets grown in contact of the ERM network of *R. irregularis* (and/or other strains) to different concentrations of exogenous ABA could be performed (Herrera-Medina et al. 2007), in order to determinate the influence of this hormone on the early mycorrhization of the rubber plantlets.

Soil microorganisms. Pseudomonas fluorescens, *Azospirillum brasilense*, and *Trichoderma harzianum* have been shown to favor the production of roots and the *ex vitro* acclimatization of tea plantlets produced *in vitro* (Thomas et al. 2010). The MDP *in vitro* culture systems was already used to test the effects of the *in vitro* co-culture *of S. tuberosum* plantlets inoculated with *R. irregularis* and *T. harzianum* (de Jaeger et al. 2011). Thus, a similar microcosm system could be adapted to the *H. brasiliensis* plantlets associating root-stimulating microorganisms and AMF.

Plantlets genetics, root anatomy and age. During the course of our experiments, we distinguished the different types of roots previously described for *H. brasiliensis* by Le Roux and Pagès (1994; 1996) and Putranto et al. (2012) at the very early stages of growth *in vitro*. In addition, the close relationship

between the age, the anatomy, and the type of roots with the colonization by AMF (Hepper 1985; Zadworny and Eissenstat 2011) was confirmed.

Furthermore, we observed that the secondary roots produced by *H. brasiliensis* in autotrophy (and not the pre-existing old roots formed after the embryo germination) were frequently colonized by *R. irregularis*. Thus, we could hypothesize that the different types of roots formed during the first weeks of development of the rubber plantlets *in vitro* have different susceptibility to the mycorrhization. To verify this hypothesis, the dynamics of root generation in the presence (and in the absence) of the AMF should be conducted.

Interestingly, the transcriptome of the root system of *H. brasiliensis* clone PB 260 plantlets was recently published (Duan et al. 2013). With this information, the genes involved in the lateral root production and in the hormonal pathways that may control the mycorrhization process (López-Ráez et al. 2010) could be identified and further used for genetic transformation studies.

For instance, Leclercq et al. (2012) transformed *H. brasiliensis* lines over-expressing a cytosolic HbCuZnSOD gene to study specific gene expression patterns under water deficit stress. Interestingly, they also noticed that these transformed plantlets had highly developed root systems (Montoro, *personal communication*). Thus, rubber clones with accelerated rhizogenesis could be regenerated and further utilized in the analysis of their mycorrhizal capabilities.

Root exudates. It is important to remember that the production of roots and their subsequent colonization may be influenced by the presence of endogenous or exogenous substances accumulated in the culture medium. It could be useful to determine if the *H. brasiliensis* plantlets produce secondary metabolites with antifungal/anti-AMF activity as suggested in **Chapter 4**. To attain this objective, the MDP *in vitro* culture systems could be used to measure the impact of plantlets derived substances with antibiotic and/or antifungal properties on the root colonization of *H. brasiliensis* and/or *M. truncatula* plants *in vitro*. The effect of the following substances could be assessed:

- Crude *H. brasiliensis* root exudates (Vierheilig et al. 2003)
- Latex (Kanokwiroon et al. 2008)
- Isolated latex components with antifungal capacities contained in the laticifers, such as the hevein-like antimicrobial peptides (Martin

1991; Van Parijs et al. 1991; Jekel et al. 2005; Taira et al. 2005; Odintsova and Egorov 2012; Stotz et al. 2013)

In an alternative experiment, the detection of specific hevein-like antimicrobial peptides (such as hevein of chitinases) exuded during the growth of *H. brasiliensis* seedlings in the MDP *in vitro* culture systems could also be quantified. In that case, a dynamic of root colonization could be paralleled with the detection of the specific compound(s) exuded in the culture medium during the time course of the experiment. Specific protein detection techniques as Western blot could be used (using the suitable antibody; Laukkanen et al. 2003).

Furthermore, the *H. brasiliensis* (or *U. dioica*) colonization by *R. irregularis* could involve the induction of plant defense genes (Gallou et al. 2010), responsible for the production of antifungal molecules and/or other defense mechanism(s). Thus, the information recently published by Duan et al. (2013) about the root transcripts of *H. brasiliensis* could also be exploited to study the molecular regulation of defence responses of the rubber roots to the presence of AMF and/or root pathogens such as *R. lignosus* and *P. noxius*.

Improvement of the performance of the plantlets during their acclimatization in the nursery, and successive transplantation to the field, by *in vitro* association with AMF

To establish the impact of the *in vitro* association with AMF at transfer to the nursery and/or field conditions, at least two factors should be considered. First, we cannot discard that AMF strains native of rubber soils (in natural stands or in plantations) could be more effective than the strain used during this research. AMF are usually described as generalist microorganisms capable to establish a functional symbiosis with several plants, and different works suggest that there is no partner specificity (Smith and Read 2008). However, recent molecular studies have suggested that a degree of partner selectivity exists (Davison et al. 2011). We choose *Rhizophagus irregularis* (formerly *Glomus intraradices*) because it is a species that has been largely studied (Stockinger et al. 2009). Furthermore, the strain MUCL 41833 is perfectly adapted to growth in the MDP *in vitro* culture system, and has been successfully used to colonize the roots of several plants *in vitro* (CESAMM team 2012).

However, the use of AMF strains sampled from rubber tree plantation soils could be useful to evaluate if native AMF strains can induce different responses of the rubber plantlets when inoculated *in vitro*. We do not discard the hypothesis that other AMF (e.g. native AMF) can be faster than *R. irregularis* MUCL 41833 to start the *in vitro* root colonization of the rubber plantlets. In addition, the screening of native AMF strains can be useful to select the better isolates capable to improve the resistance of the rubber plants to biotic or abiotic stresses during the acclimatization in the nursery, and/or positive growth responses. All depends on the purposes of the treatment with AMF.

Secondly, the growth promotion and the improvement of the physiology of *H. brasiliensis* plantlets colonized *in vitro* should be validated *in vivo*. After obtaining an uniform group of successful *in vitro* mycorrhized *H. brasiliensis* plantlets, the time required for reach the optimal size for the transfer to the field of mycorrhized plantlets should be compared with control plantlets (without AMF) in greenhouse and/or nursery experiments. Then, establishment of experimental plots in the field (i.e. in natural rubber plantations) for several months will be necessary to evaluate the performance of the *in vitro* mycorrhized material in field conditions. The vegetative development (e.g. trunk girthand) or the production of latex of the *in vitro* produced trees in comparison with the clones produced by traditional budding could be quantified (Carron et al. 2009).

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Overview of the scientific achievements

1) Research articles

Sosa-Rodriguez T, Dupré de Boulois H, Granet F, Gaurel S, Melgarejo LM, Carron MP, Declerck S (2013) *In vitro* mycorrhization of the rubber tree *Hevea brasiliensis* Müll. Arg. In Vitro Cell Dev-Pl 49:207-215

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Sosa-Rodriguez T, Dupré de Boulois H, Granet F, Gaurel S, Declerck S. Effect of activated charcoal and rooting of the taproot on the *in vitro* mycorrhization of the rubber tree *Hevea brasiliensis* Müll. Arg. *Submitted*

2) Conference participation

Sosa-Rodriguez T, de la Providencia I, Granet F, Gaurel S, Melgarejo LM, Carron M-P, Declerck S. 2012. . *In vitro* mycorrhization of autotrophic grown *Hevea brasiliensis* plantlets. 17th Symposium on Applied Biological Sciences. Leuven, Belgium

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3) Teaching

- Supervisor during the "International training on *in vitro* cultivation of arbuscular mycorrhizal fungi: Plant systems training". 2012. HAM-P system set-up session

- Supervisor during the "International training on *in vitro* cultivation of arbuscular mycorrhizal fungi: Plant systems training". 2010-2011. Host preparation techniques session (Root Organ Cultivation, micropropagation of potato, seed's disinfection)