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Exploration of model systems to study mycorrhizal symbiosis on roots of forest trees

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UNIVERSITÉ
DE LORRAINE



FACULTY OF SCIENCE AND TECHNOLOGIES

Exploration of model systems to study mycorrhizal symbiosis on roots of forest trees

Master's thesis

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Master in biotechnologies, microbiology, food, nutrition and environment, option for
environmental and sanitary microbiology

January - July 2018

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Prof. Dr. T. Bisseling



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Abbreviations

ADP: Adénosine diphosphate

ATP: adenosine triphosphate

BioMANE: Biotechnology, Microbiology, Food, Nutrition and Environment

DNase: Désoxyribonucléase

dNTP: Deoxynucleotide phosphate

EcM: ectomycorrhizal fungi

IBA: indole-3-butyric acid

KOH: Potassium hydroxide

MFS: major facilitating superfamily

MMN: Melin-Norkans medium

MS: Murashige & Skoog

PhT: phosphate transporter

PHS: phosphate-H⁺ symporter

Pi: inorganic phosphate

SOM: soil organic matter

RNA: ribonucleic acid

P20: Pachlewski medium 20

PBS: Phosphate-buffered saline

PCR: polymerase chain reaction

PDA: potato dextrose agar

qPCR: quantitative polymerase chain reaction

RT-PCR: real time polymerase chain reaction

SH: Schenk Hildebrandt

WGA: Wheat Germ Agglutinin

WPM: woody plants salt mixture

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I. Introduction

1. Laboratory of Molecular Biology

To close my tenth semester and thus my second year of the BioMANE Master, Biotechnology, Microbiology, Food, Nutrition and Environment, within the Faculty of Science and Technology at Vandœuvre-lès-Nancy, I had a chance to make an internship in a research laboratory. I have chosen the Laboratory of Molecular Biology of Wageningen University in the Netherlands. I'm interested to studying more the interaction between plant and micro-organism. This lab is famous in this field; I expect to learn a lot. This University is the only University in the Netherlands that focuses specifically on "healthy food and living environment" and plant science. Wageningen's status as an institute of higher education was legally ratified, and it became the National Agricultural College on 9 March 1918. Laboratory of Molecular Biology belongs to Plant Science Department.

Since its origin in 1972, the lab of Molecular Biology has made major contributions to the development of molecular biological technology for fundamental and applied research. One of the mega- projects which made this lab famous is legume-rhizobium symbiosis. Legumes can establish a mutually beneficial symbiotic interaction with bacteria collectively referred to as rhizobia. This symbiosis culminates in the formation of a new root organ called nodule. In the nodule rhizobia accommodate inside of plant cell, where they differentiate and become able to convert atmospheric nitrogen into ammonium which can be used by plant as a nitrate resource. As reward, bacteria get carbon from a plant. Now three topics related to this project are still running in the lab, they are 1) bacterial invasion of plant cells (creation of interface between plant and bacteria), 2) nodule organogenesis (NIN transcription factor is at the base of nodule initiation), 3) genetic and comparative genomic studies by using tropical trees, *Parasponia* species (Cannabaceae) , the only non legumes that can establish nitrogen fixing nodules with rhizobium, and its non-nodulating relative *Trema*.

Recently, the lab initiated work on ectomycorrhizal symbiosis. This is a topic I am interested in, before I have already done some work on ectomycorrhizal fungi.

2. Project

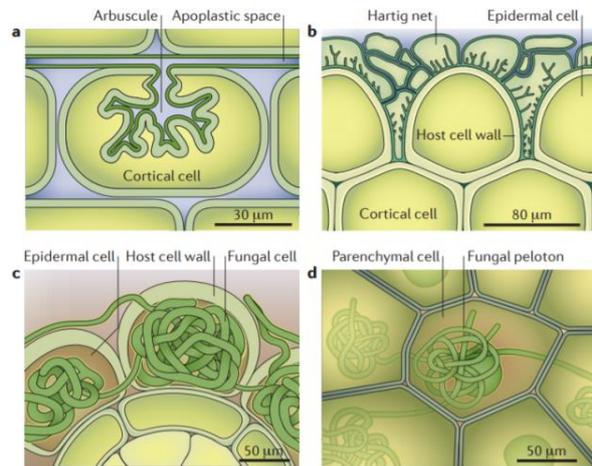
The study of the ectomycorrhizal symbiosis has just been initiated in lab of Molecular Biology. Since the existing models like a hybrid poplar, *Poplar trichocarpa* have certain (advantages and) disadvantages, my task was to compare some models and search for the best (or new) model to study the mycorrhizal symbiosis.

The mycorrhizal symbiosis is the commonest and most important mutualistic association of roots and soil fungi (Martin et al., 2007). The fungal mycelium delivers soil minerals, particularly phosphorus and nitrogen, to the host roots; the plants reward them with photo assimilates (Landeweert et al., 2001; Read and Perez-Moreno, 2003). In addition, these fungal mycelia link one plant to another, transferring nutrients not only among fungi but also from plant to plant, shaping the biological makeup of whole communities (Read and Perez-Moreno, 2003). Most of terrestrial plants, about 95%, are colonized by mycorrhizal fungi (Martin et al., 2007).

The classification of mycorrhizal fungi mostly depends on the structure formed inside or outside the plant roots and on the taxonomic position of the partners. Traditionally, the variety of mycorrhizal associations is divided into two main types, according to their ability to colonize the root cells: ecto- and endomycorrhizae; they form two main different types of plant-fungus interface (Balestrini and Lumini, 2017). However, there are more types of mycorrhizal symbiosis: arbuscular mycorrhiza, ectomycorrhiza, ericoid mycorrhiza and orchid mycorrhiza (Figure 1) (Martin et al., 2016; Read and Perez-Moreno, 2003).

Figure 1: The common types of mycorrhizal symbiosis (Martin et al., 2016)

- (A) Arbuscular mycorrhiza,
- (B) Ectomycorrhiza,
- (C) Ericoid mycorrhiza and
- (D) Orchid mycorrhiza



The ectomycorrhizal associations are youngest form of mycorrhizal symbioses. There are two main phyla of these fungi: *ascomycetes* (truffles) and *basidiomycetes* (agaric, bolets) (Martin et al., 2001, 2016; Nehls et al., 2001; Read and Perez-Moreno, 2003). And the associated with these fungi trees belong to families: *Fagaceae* (beech, oaks), *Pinaceae* (pines) or other trees such as *Eucalypts*, *Dipterocarps* and *Poplar*(Martin et al., 2016; Nehls et al., 2001).

The formation of mycorrhizal roots is considered as the most widespread response to increase phosphate acquisition by plants (Plassard et al., 2011). The phosphorus is one of the most important nutrients for plants (Zhao et al., 2003). But phosphorus concentration in soil rarely exceeds 10 μ M, which limits plant growth (Tan et al., 2012). Moreover, organic phosphate in the soil is not directly available to plants and must first be mineralized to inorganic phosphate (Pi). So, the primary source of phosphorus for plants is the Pi (Smith et al., 2003). The Pi is utilized by a plant for the synthesis of nucleic acids, phospholipids, ATP and ADP (Glassop et al., 2007). Plants adapt their morphologies, biochemistry and symbiotic strategies to increase the acquisition of Pi and/or improve the efficiency of internal utilization of Pi (Smith et al., 2003).

Poplars are among a few tree genera that can develop both, ectomycorrhizal (ECM) and arbuscular (AM) associations, simultaneously or not (Aguillon and Garbaye, 1990; Karliński et al., 2010). *Castanea mollissima* (Chinese chestnut) belongs to family *Fagaceae*. It is grown as an ornamental tree and/or for its edible nuts in China and Korea. It is noted for

its resistance to chestnut blight (*Endothia parasitica*) which is a fatal fungal bark disease. This disease has nearly wiped out the native American chestnut (*Castanea dentata*). It has already been described that *C. mollissima* can develop symbiosis with ECM (Geng et al., 2009). However, symbiosis with arbuscular mycorrhiza has not yet been reported.

Both *Parasponia* and its close relative *Trema* can develop symbiosis with arbuscular mycorrhiza and *Parasponia* also with rhizobia. However, nothing is known about their ability to form symbiosis with ECM. If they could do so it would be the perfect model plants to compare 3 different type of symbiosis.

In order to find a good model plant system for ectomycorrhizal symbiosis studies I explored to what extent the disadvantages of the existing poplar system can be overcome in model plant *Parasponia* and its close relative *Trema*, and also chestnut as a model for commercial applications of ectomycorrhizal fungi as growth promoter.

In the present work:

- I successfully propagated a poplar hybrid by “cuttings”, inoculated its roots with ECM and tested a few transformation methods.
- Two phosphate transporter *PtPT1.9* and *PtPT1.12* genes of *Populus tremula x alba* were amplified, cloned in pENTR-TOPO and sequenced. Their expression during ectomycorrhizal colonization with three different fungal species was analyzed by qPCR.
- I studied the morphology of ectomycorrhizal symbiosis between three ectomycorrhizal fungi (*Laccaria bicolor* S238N, *Scleroderma polyrhizum* and *Cenococcum geophilum*) and *C. mollissima* in comparison with *Populus tremula x alba*.
- I tested the ability of Chinese chestnut roots to make symbiosis with arbuscular mycorrhizal fungi *Rhizophagus irregularis*.
- I developed for *Parasponia* and *Trema* inoculation procedure with ECM and showed that *Trema* is able to develop ectomycorrhizal symbiosis but *Parasponia-not*.

II. Results and discussion

1. Morphology of ectomycorrhizal roots of *Castanea mollissima* in comparison with *Populus tremula x alba* 717 INRA clone

Laccaria bicolor has ability to produce ectomycorrhizal symbiosis on roots of many woody plants. Formation of this symbiosis was described in details for a hybrid poplar, *Populus tremula x alba* (Baum et al., 2002; Desai et al., 2014; Felten et al., 2009; Martin et al., 2008; Müller et al., 2013; Plett et al., 2011; Vayssières et al., 2015). Here, we tested whether *Laccaria bicolor* can also establish symbiosis with roots of *Castanea mollissima*.

First of all, we needed to get both, plants and fungi, grow well in the lab conditions. Growth conditions for the *in vitro* propagation of different poplar species had already been available (Müller et al., 2013). By using them (see M&M) the propagation of poplar was successful, the roots were well developed after 15 days of growth (Figure 2).

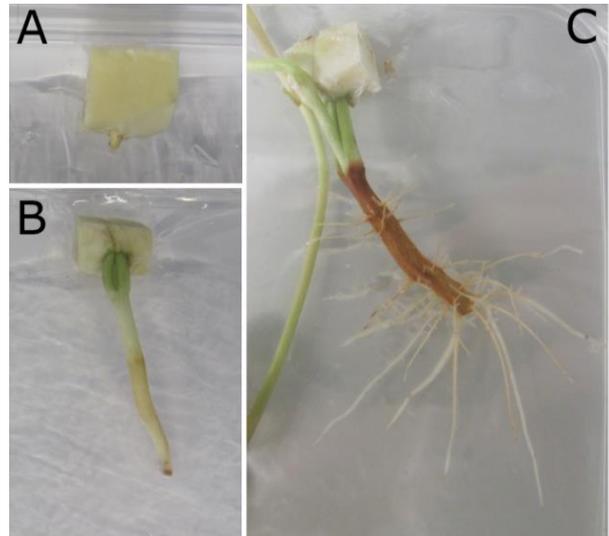


Figure 2: Growth of poplar roots on rooting medium

Chestnut forms a long and woody main root very fast, it was impossible to inoculate roots with ectomycorrhiza on a plate. The solution was simple, to cut main root at 6 day after germination. Then plant forms many thin anchor roots within 2weeks (Figure 3).

Figure 3: Growth of *C. mollissima*

(A) Seed after sterilization, removing of seed coat and cutting off endosperm around embryo; (B) the cut main root after 6 days of growth; (C) anchor roots .



The propagation of three types of fungi which were used in this study was also successful (Figure 4). Fungi grew on a cellophane to co-cultivate plant and fungus (“sandwich” culture system, see M&M).



Figure 4: Culture of ectomycorrhizal fungi on P20 medium on a cellophane

(A) *Scleroderma polyrhizum*, (B) *Laccaria bicolor* S238N, (C) *Cenococcum geophilum*

The different media for culturing of ectomycorrhizal fungi were used, and the best one is a modification of the Melin-Norkans culture medium (MMN). This medium allowed better fungal growth than the Potato Dextrose Agar (PDA) medium (Barros et al., 2006). This medium was used for all three different fungal species. It is important to refresh fungal culture time by time by transferring of portion of mycelium to a new plate.

The co-cultivation of *Populus tremula* x *alba* and *C. mollissima* with three types of mycorrhizal fungi resulted in mycorrhization of roots (Figure 5). The fungus forms around the roots a structure called mantle. Both, poplar and chestnut, showed a good growth on cellophane forming many short lateral roots.

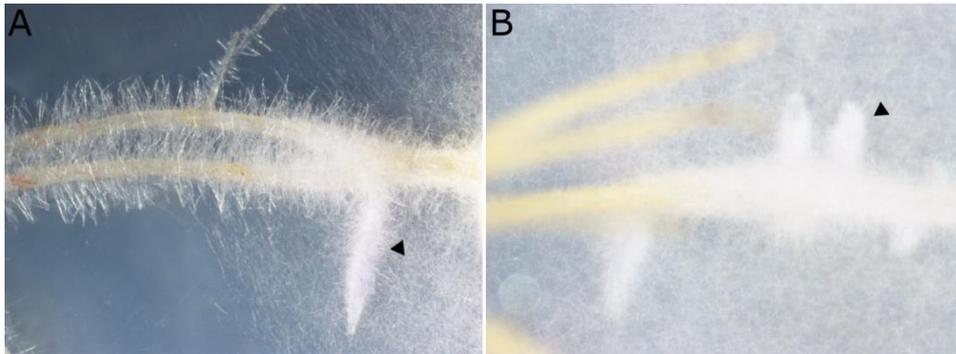
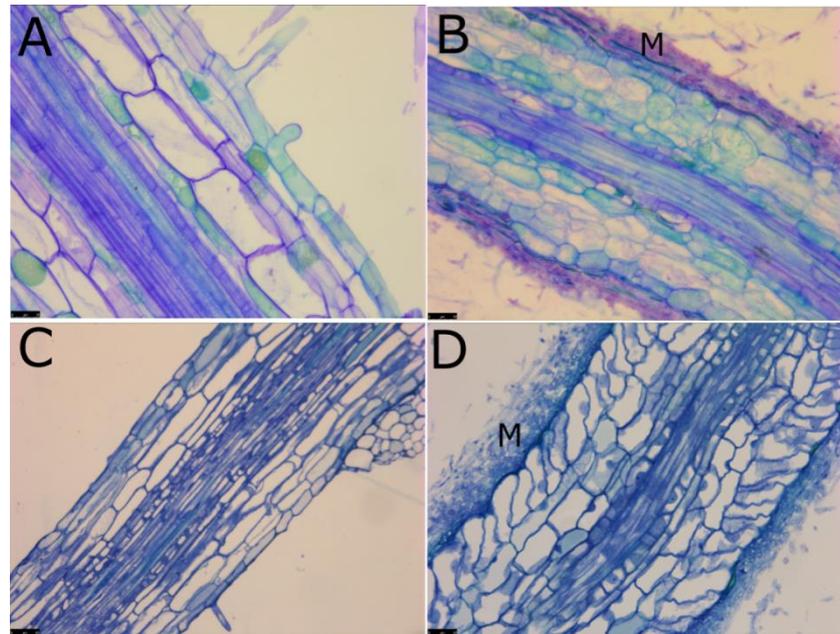


Figure 5: Ectomycorrhizal roots after inoculation with *Laccaria bicolor* S238N

(A) poplar roots and **(B)** chestnut roots

To get inside of roots and compare organization of ectomycorrhizal roots of poplar and chestnut, roots were fixed in paraformaldehyde, embedded in plastic (Technovit7011), sectioned, stained with Toluidin Blue and analyzed under microscope (Figure 6). Structurally, ectomycorrhizal roots are characterized by the presence of a fungal mantle that envelops host roots and a Hartig net that surrounds root epidermal and/or cortical cells and provides a large surface area for resource exchange. In plastic sections (Figure 6) mantle can be seen but Hartig net is not detectable. However, morphology of ectomycorrhizal root is certainly changed. First, there are no root hairs in inoculated roots in both, poplar and chestnut. Second, shape and size of cells especially epidermal and cortical are altered; cells become smaller and less elongated in both plants. This is probably effect of many molecules such as auxin, ethylene produced by fungi and also by plant (Martin et al., 2001; Felten et al., 2009).

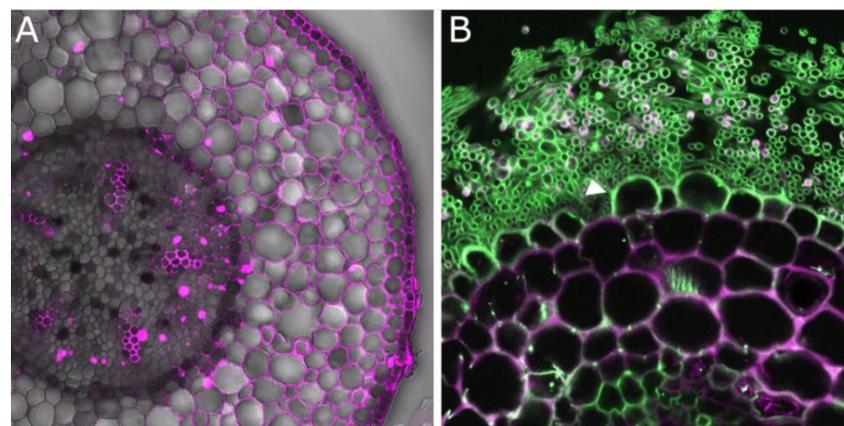
Figure 6: Longitudinal sections of poplar and chestnut roots non-inoculated and inoculated with ectomycorrhizal fungi.



(A) Non-inoculated poplar root; **(B)** poplar root inoculated with *Laccaria bicolor* S238N; **(C)** Non-inoculated chestnut root; **(D)** chestnut root, inoculated with *Scleroderma polyrhizum*; M= mantle

To visualize a Hartig network, ectomycorrhizal roots were stained with UVitex 2B and counterstained with Propidium iodide (Felten et al., 2009). UVitex 2B dye targets chitin of the fungal cell walls. Propidium iodide stains root cell membranes. In Figure 7 mantle surrounding root and Hartig net can be detected. Epidermal cells have oval shape and hyphae penetrated space between them. This picture is very much the same as in case of poplar iodide (Felten et al., 2009). So, there is no difference on morphological level in the development of ectomycorrhizae a symbiosis between these two plants.

Figure 7: Colonization of chestnut roots by *Laccaria bicolor* in an *in vitro* “sandwich”



(A) cross-section of control root; **(B)** cross-section of ectomycorrhizal root; Magenta, propidium iodide; green, Uvitex2B

2. Gene expression analysis of phosphate transporter 1.9 and 1.12 in *Populus tremula x alba* during ectomycorrhizal colonization

Different Pi transporter genes control the transport of the Pi in different tissues of mycorrhizal and non-mycorrhizal plants during different growth conditions and developmental stages (Dong et al., 1999). Generally, the phosphate transporters are classified into 4 families; PHT1, PHT2, PHT3, and PHT4 (Zhang et al., 2016). PHT1 family is involved in the Pi uptake from the soil and distribution within plants and also members of this family respond to mycorrhizal colonization (Loth-Pereda et al., 2011). This family contains 12 members. The expression of *PtPT1.9* and *PtPT1.12* genes is highly up-regulated in presence of ectomycorrhizal fungi whereas the *PtPT1.1* and *PtPT1.11* expression is strongly down-regulated (Loth-Pereda et al., 2011).

Here, I studied expression of PHT1.9 and PHT1.12 in *Populus tremula x alba*. The idea was to confirm up-regulation of these genes during mycorrhization using this time three different ectomycorrhizal species to see whether they all can trigger the expression of these phosphate transporters, and later perform ViewRNA *in situ* hybridization on ectomycorrhized root sections. This could be quite interesting to see dynamics of plant responses to invasion of fungi.

First, the amplification of these two genes from cDNA of ectomycorrhizal roots was conducted by PCR. PCR fragments purified from a gel were cloned in pENTR-TOPO vector (Invitrogen) and sequenced because the sequences in the database are quite variable. Based on these DNA sequences RNA *in situ* probes were ordered in Thermo Fisher Scientific. RNA *in situ* will be performed in the nearest future.

Also, qPCR primers were designed and qPCR was performed for the control and ectomycorrhized roots in 3 biological replicas (Figure 9). House-keeping gene *Actin 9* was

used as a reference gene. The *SUT* (*sucrose transporter*) gene is constitutively expressed in roots and a good quality marker for the isolated RNA (Figure 9A).

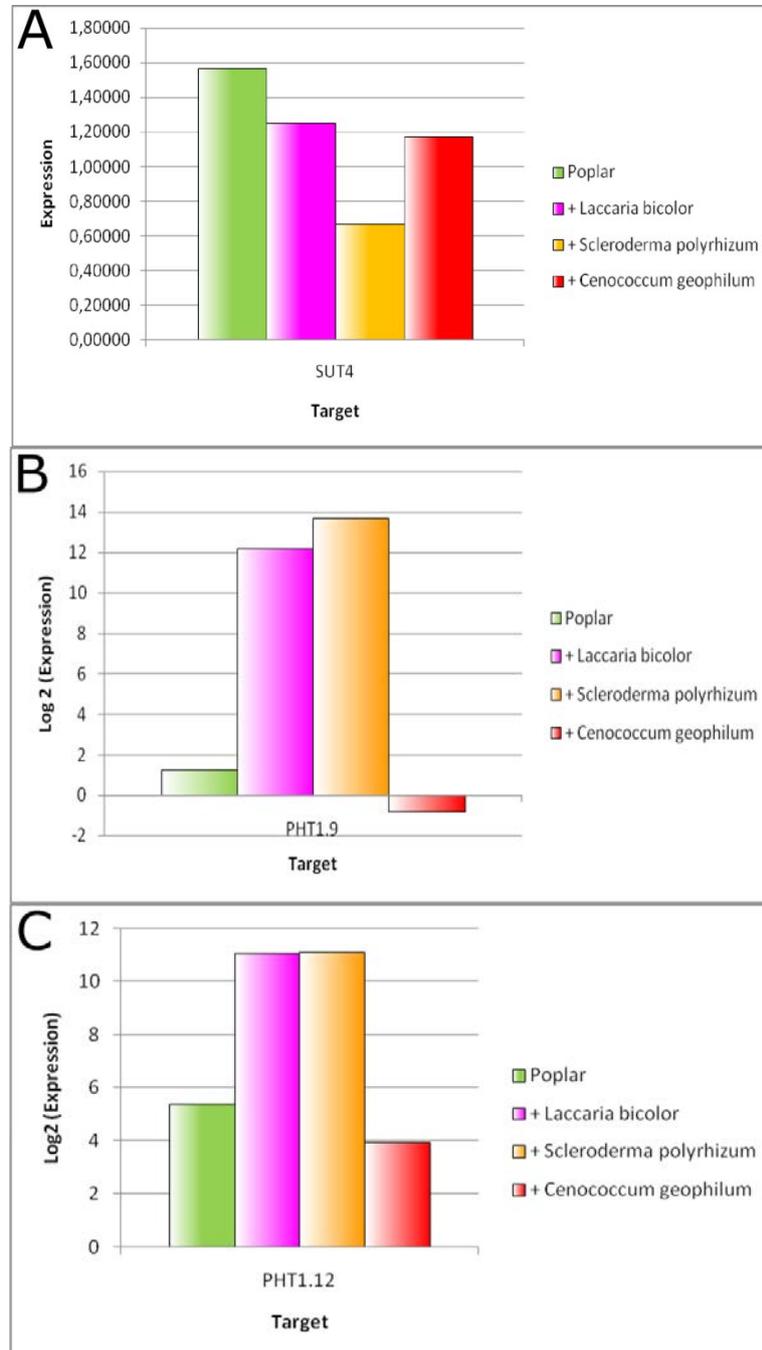


Figure 8: Expression level of the *PHT1.9* and *PHT1.12* in *Populus tremula x alba* roots colonized by different ectomycorrhizal fungi, *Laccaria bicolor*, *Scleroderma polyrhizum* and *Cenococcum geophilum*.

(A) qPCR results of *SUT4*, (B) qPCR results of *PHT1.9*, (C) qPCR results of *PHT1.12*

The qPCR results indicated that the *PHT1.9* and *PHT1.12* expression during mycorrhization depend on fungal species. *Laccaria bicolor* and *Scleroderma polyrhizum* both activate expression of these genes but *Cenococcum geophilum* does not. So, probably there is species specific regulation of phosphate transporter genes. *Cenococcum geophilum* is a member of a family different from the other two fungi. It belongs to the clade ascomycetes while *Laccaria bicolor* and *Scleroderma polyrhizum* belong to the clade basidiomycetes. This can be the reason that evolutionary these fungi have different signaling pathways and activate different sets of genes in plants. *Laccaria bicolor* belongs to the Agaricales and *Scleroderma polyrhizum* is part of the Boletales. But it seems that these phosphate carriers are common between those two subfamilies. Indeed, *Paxillus involutus* is part of the same subfamily as *Scleroderma polyrhizum* and shows the same pattern of expression for these phosphate transporters as *Laccaria bicolor* (Loth-Pereda et al., 2011).

The other explanation could be the collecting of plant material was done not at the same stage of symbiosis. The symbiosis and therefore the formation of the Hartig net is set up more quickly by *Laccaria bicolor* and more slowly by *Cenococcum geophilum*. If the harvest is done when the mantle is formed but not the Hartig net, this could affect the results. This experiment needs to be repeated with roots mycorrhized to the same extend.

A final hypothetical explanation is influence of the phosphate concentration in the growth medium. In our experiments the phosphate concentration is a high compared to what can be found in the natural environment. It is therefore possible that during symbiosis phosphate transport is not necessary between the two organisms. Indeed, it has been shown that the level of phosphate in soil influences the transcription of some phosphate transporter genes (Zhang et al., 2016). Experiments were set up with different phosphate concentrations to obtain more answers to our questions.

3. Testing the ability of Chinese chestnut roots to make symbiosis with arbuscular mycorrhizal fungi *Rhizophagus irregularis*

There are two types of mycorrhizal symbiosis, ectomycorrhizal and arbuscular mycorrhizal symbiosis. Some plants can develop both types of symbiosis separately or simultaneously (Aguillon and Garbaye, 1990; Karliński et al., 2010). Chinese chestnut,

C. mollissima can form ectomycorrhizal symbiosis with the truffle, *Tuber indicum* (Geng et al., 2009) and in our study with *Laccaria bicolor*. Here I tested whether this tree can also make symbiosis with the arbuscular mycorrhiza.

After 7-9 weeks after inoculation with arbuscular mycorrhiza inoculated roots were harvested, and stained with Trypan Blue (Figure 9A). This dye stains dead cells or tissue in blue. It is commonly used for the staining of fungal hyphae (Loth-Pereda et al., 2011). After this staining we could detect hyphae but not arbuscules. Next, mycorrhized roots were fixed, embedded in plastic, sectioned and stained with Toluidine Blue. This allows to detect the mycelium penetrating the cortical cells (Figure 9B). A final staining was done with WGA which targets chitin of fungal cell wall (Kerley and Read, 1997). This staining helps to detect the arbusculars inside of cells (Figure 9C).

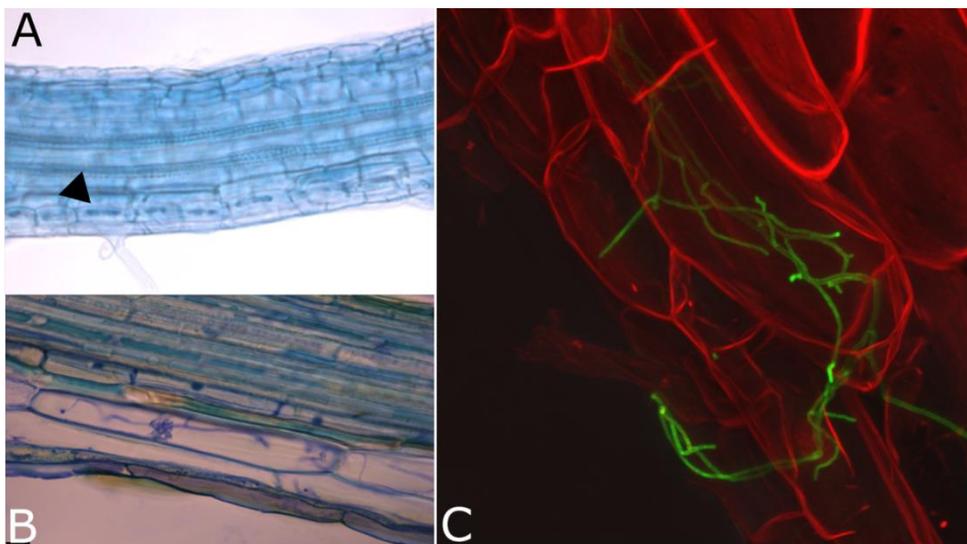


Figure 9: Chestnut roots inoculated with arbuscular mycorrhizal fungi *Rhizophagus irregularis* and stained with different dyes to visualize mycorrhiza

- (A) Staining by Trypan blue,
- (B) Staining by Toluidine blue,
- (C) Staining by WGA and propidium iodide

4. Can *Parasponia andersonii* and *Trema* develop an ectomycorrhizal symbiosis?

Previous studies in the lab of molecular biology have shown that two tropical tree species, *Parasponia* and *Trema* can form arbuscular mycorrhiza. In addition *Parasponia* represents the only non-legume species to form root nodules by interacting with nitrogen-fixing rhizobia. The genus *Parasponia* represents a clade of three species that is phylogenetically embedded in the closely related *Trema* genus (van Velzen et al., 2018). These two plant species, their genomes are sequenced, are fast growing trees and an efficient and rapid transformation protocol for *Parasponia* was set up in the lab of molecular biology (van Zeijl et al., 2018). The transformation for *Trema* is feasible and a protocol is optimizing as well. These allow to study the mechanism for ectomycorrhizal symbiosis formation, if *Parasponia* and/or *Trema* could form ectomycorrhiza. Here an in vitro culture experiments were set up for *Parasponia* and *Trema* with ectomycorrhizal fungi. Experiments were carried out with a species of *Parasponia*, *Parasponia andersonii* and three species of *Trema*, *Trema levigata*, *Trema tomentosa* and *Trema orientalis*.

We inoculated *Parasponia andersonii* with *Scleroderma polyrhizum* in the sandwich system. After 15 days, the plant growth close to the fungi but the formation of the mantle is not carried out during the contact (Figure 10AB). Also, during ectomycorrhizal symbiosis, both organisms synthesize many molecules (auxin, ethylene) that produce lateral root growth. In this case, the number of secondary roots on ectomycorrhizal plates was the same than that on control roots without any inoculation (Figure 11C). Though this the possibility that *Parasponia andersonii* can form ectomycorrhizal symbiosis cannot be ruled out, because this plant has some difficulty in developing roots on agar associated with a cellophane membrane. Other devices, such as a pot containing perlite in which *Parasponia* grows very well, will be used to replace the sandwich system to further test whether *Parasponia* interacts with ectomycorrhiza.



Figure 10: *in vitro* culture system of *Parasponia andersonii* and *Scleroderma polyrhizum*.

(A) Co-culturing of *Parasponia* and *Scleroderma*, **(B)** Focus on root tip, **(C)** Control

The interaction between ectomycorrhiza and *Trema* was studied in the same way as *Parasponia*. Different combinations of ectomycorrhiza and *Trema* species were tested. The ectomycorrhizal fungus allowing making a coat during the growth of *Trema* plant was *Laccaria bicolor* therefore studies with this fungus was analyzed in detail. For three types of *Trema*, *Trema levigata*, *Trema tomentosa* and *Trema orientalis*, we can notice that the roots of *Trema orientalis* responds to fungal stimuli, since an increase in the number of secondary roots is observed. The growth of the mycelium very close to the root could be noticed in all 3 *Trema* species (Figure 11ABC), suggesting an ectomycorrhizal symbiosis can be formed. To validate this, other experiments like embedding technique and/or dye staining must be taken in place.



Figure 11: *in vitro* culture system of *Trema*'s species with *Laccaria bicolor*.

(A) Co-culturing of *Trema levigata* and *Laccaria bicolor*, **(B)** Co-culturing of *Trema tomentosa* and *Laccaria bicolor*, **(C)** Co-culturing of *Trema orientalis* and *Laccaria bicolor*

A host preference for *Laccaria bicolor* likely exists. In case of *Trema orientalis*, on ectomycorrhizal plates, plants grow much better than the other two species, compared to the control (Figure 12A). The root portion has many lateral roots as well as the beginning of lateral root secondary root growth (Figure 12B). On some *Trema orientalis* roots, we observe the presence of mycelium very close, forming a structure similar to the fungal mantle in poplar or Chinese chestnut (Figure 12C). The morphology of this fungal mantle will be studied in the future.



Figure 12: *in vitro* culture system of *Trema orientalis* and *Laccaria bicolor*

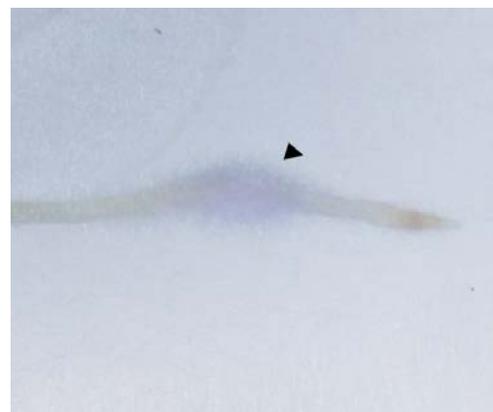
(A) Global observation, **(B)** focus on roots with many lateral roots, **(C)** focus on roots with mycelium close of the root.

Collectively, *Trema orientalis* could become the model to study how an ectomycorrhizal symbiosis is established under lab conditions. Many works on this plant are in progress. Sterilization, germination and propagation are optimized and perfectly performed protocols. Transformation by *Agrobacterium* tends to be easily implemented and in this way mutants could be generated rapidly. This will shed light on molecular study on this symbiosis.

5. Conclusion

In conclusion, the objective of my internship was to develop an efficient interaction between different forest trees and fungi to study the mycorrhizal symbiosis. For this purpose, I studied the morphology of the ectomycorrhizal symbiosis in a series of plants, *Populus tremula x alba*, *Populus trichocarpa*, *Castanea mollissima*, *Parasponia andersonii* and 3 *Trema* species. The poplar hybrid is a known model to study ectomycorrhizal symbiosis and a positive regulation of phosphate transport genes, *PHT1.9* and *PHT1.12*, in the presence of ectomycorrhizal fungi had been demonstrated. The advantage of this tree model is that the symbiosis is established quickly and easily (Part I). Although the *Agrobacterium* mediated transformation for this tree has already been demonstrated, to study the molecular mechanism for the symbiosis formation, however, is quite a challenge in this hybrid. Because this is the organism originated from a cross between two individuals, *Populus tremula* and *Populus alba*. It therefore has genetic characteristics from both parents and some genes are duplicated. A homozygous *Populus trichocarpais* shown to form ectomycorrhiza, whereas it is difficult to be transformed.

Figure 14: *In vitro* culture system of *Populus trichocarpa* and *Laccaria bicolor*.



Meanwhile, the possibility of Chinese chestnut could be taken into account as another model. Indeed, it is possible to form a symbiosis with *Laccaria bicolor* and the transformation protocol for this tree has been made available. In addition, the ability of Chinese chestnut roots to form a symbiosis with arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, has been revealed here. The comparison of these two types of symbiosis could be done on this model. The disadvantage of this plant is that the propagation is not easy. In addition, sequencing of the genome is not yet efficient.

In my report, other tree models, at least *Trema orientalis*, has been highlighted to form symbiosis with *Laccaria bicolor* (Figure 11). *Trema orientalis* is not a hybrid and its genome was sequenced and therefore is feasible to perform genetic modification to study the mechanism for mycorrhizal symbiosis formation.

I. Materials and methods

1. Plant and fungi material, their growth conditions

Some of the experiments were done on hybrid poplar (*Populus tremula* x *Populus alba*; INRA clone 717-1-B4). Plants were propagated thanks cuttings with nodes and internodes. Cuttings was first put in MS medium (*annex 1*) containing a hormone called indole-3-butyric acid (IBA) allowing growth of the root system. These cuttings were put in a growth chamber at 21°C with 16hours of light during 7days. Then, cuttings were transferred to a new MS medium without IBA. This step allows the growth of the foliar system. It's carried out under the same conditions as above. Once obtaining leaves, plantlets were transferred in tubes in glasses with the same medium. For my experiment I had need of roots of poplar plants, that's why I had made growth roots of plantlet in SH medium (*annex 2*) (Müller et al., 2013). Cellophane was added on the top of the medium for the roots don't penetrate inside of the medium. These roots were growth at the vertical position with 16h light at 21°C during 14 days. Roots were recovered with aluminum so that she doesn't have light.

The other part of the experiments is done on *Castanea mollissima*. The sterilization has been made in cleaning seeds with bleach (1-5%) with water 3 times. After, seeds coats have been removed in keeping the embryos. Then the seeds have been washing with a solution of 75% ethanol during 2min and with a solution of bleach (1-5%) during 15min. Finally, seeds have been rinsing with sterilized water for 6 times in fume hood. The sterilization is finish and for the germination, the coasts of seeds have been cutting and we have keeping emerge embryos seeds. These parts of chestnut have been put in square Petri dishes of 12cm x 12cm with woody plants salt mixture (WPM medium) (*annex 3*) and 1/2 filter paper was put on the top of the medium for roots don't penetrate in the medium and to keep the humidity. This one has been made at the vertical position in darkness at 21°C.

The vegetative mycelium of ectomycorrhizal fungi *Laccaria bicolor* S238N, *Scleroderma polyrhizum* and *Cenococcum geophilum* were maintained at 28°C on P20 medium (*annex 4*) with cellophane. Cellophane was added on the top of the medium that roots don't growth inside of the medium.

2. *In vitro* “sandwich” culture system

The transformation of poplar plants with EcM fungi was realized in P20 medium and 1 g/L of MES. MES was included to stabilize the pH and avoid stress reaction of plants. For this step, cellophane was added on the top of the medium that roots don't grow inside of the medium. Then, plantlet with roots and spot of fungi were transferred. Plugs of fungi are added thanks to a cookie cutter to always have the same size of biomass. This construct was grown at the vertical position with 16h light at 21°C during 14 days. Roots were recovered with aluminum for that they don't have light.

3. Expression studies by Real-Time RT-PCR

i. Sequencing

Different steps are required. First of all, the design of primers is realized thanks to the Aspen database. After this design, a RNA extraction (*annex 5*) and cDNA synthesis (*annex 6*) was realized, followed by a Fusion PCR (*annex 7*) and electrophoresis to check the amplification of the gene, the size and the concentration. Then cloning is carried out, for this our gene is inserted in a pENTR vector thanks to a topoisomerase then into competent cells. A verification of the presence of the gene is carried out by colony PCR. For this, a normal PCR is used and a colony of competent cells having developed is added in 10µl of sterile water (*annex 7*). Then plasmids are isolated (*annex 8*) and sent for sequencing. This one uses the method of SANGER.

ii. Quantitative polymerase chain reaction (qPCR)

To carry out the quantitative PCR, a mix is made with the sample, the primers and a mix containing dNTPs, a buffer and a Taq polymerase (SYBR Green) and the primers. We used cDNAs from different samples of poplar root colonized or not with ectomycorrhizal fungi. So we have three types of sample, poplar, poplar with *Laccaria bicolor*, with

Scleroderma polyrhizum and with *Cenococcum geophilum*. The cycle of quantitative PCR is performed as follows: 30s at 95°C, 10s at 60°C during 39 cycles and a last cycle at the end to 60°C until 95°C with an increase of 5°C every 30s. Data is retrieved from the computer and analyzed.

4. AM staining in *Castanea mollissima* roots

Concerning the inoculation of Chestnut and arbuscular mycorrhizal, the sterilization and germination was realized like before. After 7 days of culture, the germinated plants are transferred to a sterile soil containing three types of substrates, sands, ceramsite and vermiculite with a ratio of 1:1:1 per pot. Inoculation is performed with a strain of *Rhizophagus intraradices* (RI) at a concentration of 125 spores per plant. During the first three weeks of growth, the plants are sprayed with sterile water and then with 200 ml of medium ½ Hoagland with a low concentration of nitrogen. The plants are allowed to incubate for 8 weeks at 21°C and 60% humidity with (light). The plants are harvested and three techniques are performed in order to observe the arbuscular fungi. The first is staining with Trypan blue staining, the second is the completion of a embedding followed by staining with Toluidine blue and the last is staining with WGA staining. The results are observed by Normarski microscope, Leica microscope, and laser confocal microscope respectively.

5. Microscopic observation

i. Embedding of plant material (Technovit 7100)

Technovit 7100 is a plastic coating system that makes very fine cuts of samples. First, sampling was placed in fixative solution containing 4% paraformaldehyde mixed with 5% glutaraldehyde in 50mM phosphate buffer (pH 7.4) during overnight at 4°C. Sampling was washing 4 times during 30min with 1% PBS and one time during 30min with MQ water. Then, a dehydration of sampling was carried out by different concentration of ethanol (10%, 30%, 50%, 70%, 90% and 100%) at room temperature with 30min per each. Then, the plastic infiltration was performing thanks 4 ratio of solution A and 100% ethanol: 1:3, 1:1, 3:1

and solution A only. The solution A is composed of Technovit 7100, Hardener I and PED 400. All the reaction was during 3 hours at room temperature. After one night in solution A at 4°C, blocks can be realized. For this one, samples were transferred in cupules by using Pasteur pipette. The solution A was removed to add immediately polymerization solution. The polymerization solution contained solution A and Hardener II. The orientation of plant material can be realized during a short period, before the polymerization of the solution. Then, cupules were covered with parafilm to make flat surface and left during one hour at room temperature followed by one hour at 37°C. A plastic block was put in every sampling and a liquid solution of Technovit 3040 was added. The polymerization is done after 15 min. Blocks were cut with Microtome (Leica) to get section with a knife. Samplings were put on a hot plate at around 58°C and after in slide with few drops of autoclave water. Slides were dry on the same hot plate that before. A staining of slide was carried out with 0,01% Toluidine Blue during 5 min. Toluidine Blue is excellent in differentiating many cell types and tissues structures (Haskins, 1982). Slide with coloration was dry and a cover slip was put thanks three drops of Eurapal. Samplings can be observed with light microscope at different magnification (Leica).

ii. Propidium iodide and UVitex 2B staining

The formation of the Hartig net in plant can be observed by section and double staining with propidium iodide and UVitex 2B for plants and fungal structures, respectively. The technique used for this staining exactly follows the protocol of a publication (Felten et al., 2009) (*annex 9*).

i. Trypan blue staining

For this, the roots are submerged in 10% KOH and heated at 90°C for 15min. Afterwards, the roots are rinsed twice with sterile water and soaked in Trypan blue for 3 minutes and heated to 90°C. Once the staining is done the roots are placed in 85% glycerol for storage until microscopic observation by Normaski microscope.

ii. Wheat Germ Agglutinin (WGA) staining

This substance enters the plant through a treatment of 10% potassium hydroxide for 15min at 90°C (Kobae and Ohtomo, 2016). The roots are rinsed twice with sterile water and incubated for two hours in 1% PBS at pH 7.4. The roots are then incubated overnight at 4°C in PBS-WGA with a final concentration of WGA at 0.2µg/ml. Then the result is observed thanks to a confocal laser microscope. And to visualize the membrane of cells more clearly, propidium iodide staining is performed.

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Annexes

Annex 1 – Composition of Murashige & Skoog MS) medium:

Components	Quantity
Murashige & Skoog medium including vitamins	4.41 g/L
Glucose	1 g/L
MES	1g/L
Agar	5 g/L

→Adjust pH to 5.8

Annex 2 – Composition Schenk Hildebrandt (SH) medium:

Components	Quantity
Schenk Hildebrandt medium	3.18 g/L
Schenk Hildebrandt vitamins	1.81 g/L
Sucrose	25 g/L
Agar	8 g/L

Annex 3- woody plants salt mixture (WPM):

Components	Quantity
Dashing agar	7%
WPM	2.3 g/L
Sucrose	3%
pH	5.8

Annex 4 – Pachlewski medium 20 (P20 medium):

Components	Quantity
Di-NH ₄ ⁺ tartrate	0.5 g/L
KH ₂ PO ₄	1 g/L
MgSO ₄ ; 7H ₂ O	0.5 g/L
Thiamine solution 100mg/L	1 mL/L
Kanieltra solution 100x	1mL/L

Kanieltra solution 100x:

Components	Quantity
Iron (in cheated form)	6 g/L
Molybdenum	0.27 g/L
B ₂ O ₃	8.45 g/L
Manganese	5 g/L
Cupper	0.625 g/L
Zinc	2.27 g/L

Annex 5 - RNA extraction protocol by E.N.Z.A.® Plant RNA Kit Standard Protocol follow of E.N.Z.A.® Plant RNA Kit DNase I Digestion Protocol

First, tissues were collected in a 2mL tubes with stainless steel beads and freeze by dipping in liquid nitrogen. They can be stored at -80°C until use. During my experiments, I collected samples of plants with and without mycorrhization. Then, the sample is ground using a TissueLyser LT (Qiagen, Hilden, Germany) for 45 seconds with a frequency of 50 oscillations per second. And, RNA extraction were realized thanks E.N.Z.A.® Plant RNA Kit Standard Protocol follow of E.N.Z.A.® Plant RNA Kit DNase I Digestion Protocol. The protocol used is the same as that provided by the supplier. Immediately after grinding of sampling by TissueLyser LT (Qiagen, Hilden, Germany), 500µL of RB Buffer was add. RB Buffer must be mixed with 2-mercaptoethanol before use. And the sampling was vortex with a maximum speed. The lysate was transferred in Homogenizer Column and a centrifugation at 14,000g during 5 min was realized. This step allows cleaning the sampling. Then, new lysate was put in new 1.5mL tubes without the insoluble pellet. One volume of 70% ethanol was added and the mix was vortex at the maximum speed during 20secondes. After that, 700µL of sample was change to a new column called HiBind® RNA Mini Column. This column allows fixing the RNA on the column. They are a centrifugation at 12,000g during 1min. The filtrate can be discarding and the step can be repeated if there is still a sample to pass. A step with DNase was adding thanks a DNase I digestion (Qiagen). For this one, the column was wash with 250µL of RNA Wash Buffer I and centrifuged at 10,000g during 1min. Therefore, 75µL DNase I digestion mixture with RDD Buffer was put directly onto the surface of the membrane of the column. The tube was let sit at room temperature for 30min. And 250µL RNA Wash Buffer I was applied to the column during 5min. After this, a centrifugation at 10,000g during 1min was carried out. And clean two times with 600µL of RNA Wash Buffer II and

centrifugation at 10,000 during 1min. Membrane was dry before elution with a centrifugation at maximum speed. For finish, the column was placed in new 1.5 tubes and 50µL of DEPC Water at 65°C was put in directly in the column. The tube was sit at room temperature during 5min and the elution of RNA was realized with a centrifugation at maximum speed during 2min. Then a complementary DNA formation step is performed.

Annex 6—cDNA synthesis by iScript™ cDNA synthesis kit (Bio-Rad laboratories, Inc., Hercules, US)

The complementary DNA (cDNA) is a DNA synthesized from a single-stranded RNA in a reaction catalyzed by the enzyme reverse transcriptase. cDNA is often used to clone eukaryotic genes in prokaryotes. And it is derived from the mRNA, so that it contains only exons, it is without introns. The cDNA synthesis was realized thanks iScript™ cDNA synthesis kit (Bio-Rad laboratories, Inc., Hercules, US). The protocol used is the same as that provided by the supplier. This kit contained a reaction mix and a reverse transcriptase. To carry out the reaction, I add 4µL of 5X iScript reaction mix, 1µL of iScript reverse transcriptase and a variable volume of sample and water. The final volume of the reaction must be 20µL and the RNA template must be around of 1µg. Then, tubes were incubating in PCR with this protocol: 5min at 25°C, 30min at 42°C, 5min at 85°C and hold at 4°C. Once the cDNA creates a first PCR step with the previously design primers is realized. This allows amplifying the genes that we want to study later.

Annex 7: PCR with Phusion Taq and PCR with « home-made » Taq protocol

A gene amplification consists in multiplying, by successive duplications (n cycles), the quantity of gene initially present in a sample. For this purpose, primers specific to the studied gene are used. During my experiments, I amplify different phosphate transporters including PHT1.1, PHT1.9, PHT1.11 and PHT1.12. The primers were therefore used at a concentration of 10µM for this experiment. They will hybridize to the previously generated complementary DNA. Also, during my experiments, I realized two types of PCR with two types of Taq polymerase. Taq polymerase is the enzyme that makes it possible to form the complementary strand of single-stranded DNA after priming. It has the property of

withstanding very high temperatures, such as those used for amplification (up to 98 ° C) and therefore remain active throughout the PCR reaction. The first type of PCR is performed with a Phusion Taq, it is a high fidelity DNA polymerase generally used for applications in which the DNA sequence must be correct after amplification. It has a Pyrococcus-like structure fused with a processivity-enhancing domain, increasing fidelity and speed. I use this type of Taq because I subsequently sequence my PCR products to be on the transcriptomic sequence of my phosphate transporters. The second type of PCR is performed with a taq polymerase "home-made" which has a lower fidelity. This allows me to perform PCRs to verify the presence of a sample. And other products are also added during the reaction, buffer, dNTPs, sample and sterile water to complete having a final volume of 50µL. For the different PCRs, the following quantities are put in the tubes:

	Phusion PCR	Normal PCR
Reagents	Volumes (µL)	
Buffer Taq 5x	10	
Buffer Taq 10x		5
dNTP	1	
Primer 1	2.5	1
Primer 2	2.5	1
Taq phusion	1	
Taq "home-made"		1
H ₂ O	to 50	

Samples to be amplified are therefore put in the PCR in order to undergo 35 cycles of duplication, and the PCR programs are as follows:

	Phusion PCR	Time	Normal PCR	Time
Initial denaturation	98°C	0:30	94°C	5:00
Denaturation		0:10		0:30
Annealing	56°C	0:30	56°C	0:30
Elongation	72°C	2:00	72°C	2:00

Final elongation	72°C	5:00	72°C	5:00
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In fact, the initiation temperature is calculated as a function of the amount of binding between A/T and between G/C in the fragment to be amplified. Indeed, the bond between the two bases A and T contain two hydrogen bonds and the bond between the two bases G and C contain three hydrogen bonds. Each bond A and T corresponds to a degree of 2 and each bond G and C corresponds to a degree of 4. As for the elongation temperature, it is calculated with respect to the size of the gene. For PCR phusion the ratio is 30 seconds for 1000 base pairs whereas for normal PCR the ratio is 1 minute for 1000bp. In general, amplified genes are around 1500bp, to be on we use 2min. And, to verify the good amplification of the genes, electrophoresis is set up.

Annex 8: plasmid isolation

The plasmid isolation was done according to the protocol E.N.R.A.[®] Plasmid DNA Mini Kit I Spin Protocol

Annex 9: Propidium iodide and UVitex 2B staining protocol

One centimeter root tips with ectomycorrhizal were fixed during overnight at 4°C in 4% *para*-formaldehyde in phosphate buffered saline (PBS, pH 7). Roots were washes in PBS and embedded in 6% agarose. Thirty micrometer transverse sections were realized using a Vibratome (Leica). Then, sections were stained in 1% UVitex 2B (Polysciences) in PBS for 2min, washed, and then counterstained with propidium iodide (1:100 dilution). Samples were observed with a confocal scanning laser microscope equipped with an X60 oil objectives. A wavelength of 405 nm was used for UVitex 2B excitation, and emission was detected between 500 and 560 nm. Propidium iodide was excited at 514 nm with an argon laser line, and the emitted fluorescence was detected above 550 nm.

Abstract

Mycorrhizal fungi supply soil minerals, particularly phosphorus and nitrogen, to the host plants, the plants reward them with carbohydrates. During the ectomycorrhizal symbiosis *Populus tremula x alba* the phosphate transport appears to be carried out by two specific transporters, *PhT1.9* and *PhT1.12*. Some trees like poplar, chestnut and *Trema* can form symbiosis with two mycorrhizal fungi, ecto- and endomycorrhiza. It was demonstrated for the first time that chestnut can form endomycorrhizal symbiosis. *Trema* is close relative of *Parasponia* which can develop symbiosis with arbuscular mycorrhiza and also with rhizobia. *Trema* and *Parasponia* are sequenced, transcriptome data are available. *Parasponia* is easy to propagate by regeneration, transformed, and transformation of *Trema* is under very intensive investigation. All these make *Trema- Parasponia* very attractive model to study ectomycorrhizal symbiosis in the connection to two other symbioses, endomycorrhizal and rhizobial.

Keywords: symbiosis model system, ectomycorrhizal fungi, arbuscular mycorrhizal fungus, *Populus tremula x alba*, *Castanea mollissima*, *Parasponia andersonii*, *Trema*

Résumé

Les champignons mycorhiziens fournissent des minéraux provenant du sol, en particulier le phosphore et l'azote ; en échange, les plantes transfèrent des hydrates de carbone. Au cours de la symbiose ectomycorhizienne avec *Populus tremula x alba*, le transport du phosphate semble être effectué par deux transporteurs spécifiques, *PhT1.9* et *PhT1.12*. Certains arbres comme le peuplier, le châtaignier et *Trema* peuvent former une symbiose avec deux champignons mycorhiziens, et réaliser des ecto- et des endomycorhizes. Il a été démontré pour la première fois que le châtaignier peut former une symbiose endomycorhizienne. *Trema* est un parent proche de *Parasponia* qui peut développer une symbiose avec les mycorhizes arbusculaires et aussi avec des rhizobiums. Ces deux plantes sont séquencées, les données transcriptomiques sont disponibles. *Parasponia* est facile à propager par régénération, à transformer, et la transformation de *Trema* est sous étude très intensive. C'est pourquoi *Trema-Parasponia* seraient un modèle très attractif pour étudier la symbiose ectomycorhizienne en connexion avec l'étude de deux autres symbioses, endomycorhizienne et rhizobienne.

Mots-clés: système modèle de symbiose, champignon ectomycorhizien, champignon mycorhizien arbusculaire, *Populus tremula x alba*, *Castanea mollissima*, *Parasponia andersonii*, *Trema*

