UCLouvain

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Evaluation *in vitro* de l'interaction entre les microalgues *C. vulgaris* et *S. dimorphus* et la symbiose mycorhizienne de champignons mycorhiziens à arbuscules du genre *Rhizophagus*

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List of abbreviations

AM : Arbuscular mycorrhizae
IRM : Intraradical mycorrhizae
ERM : Extraradical mycorrhizae
C. vulgaris : Chlorella vulgaris
S. dimorphus : Scenedesmus dimorphus
S. acutus : Scenedesmus acutus
Sp. : specie
BF : Branching factor
ROC: Root organ culture
CTRL: control
SD: Standard deviation
MSR- : MSR medium without sugar and vitamins
MSR+G1: MSR medium with glucose (1g/L) and vitamins
MSR+G10: MSR medium with glucose (10g/L) and vitamins
MSR+S and MSR+: MSR medium with sucrose (10g/L) and vitamins
C: Carbon
N : Nitrogen
P : Phosphorus

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I. Introduction

I. Global Context

'There are more species of fungi, bacteria and protozoa in a single scoop of soil than there are species of plants and vertebrate animals in all of North America' (Stamets, 2005). Fungi are everywhere and this, since 3500 million years ago (Taylor *et al.,* 1995). Nowadays, more than 120 000 species are listed, but their number is estimated above 1.5 million (Declerck, 2018). They are keystone species, contributing in many ways to ecosystems functioning. Examples of the several services offered by fungi are the structuration of soils, improving nutrients availability, recycling nutrient and energy, assisting plant primary production, connecting them and being an intermediate in exchanges, depolluting soils, taking part of the food web, regulating population and structuring microbial communities (Fortin et al., 2016; Carlile et al., 2001). According to Stamets (2005), who takes this a little bit further; 'without fungi, all ecosystems would fail'. More specifically, mycorrhizal symbioses play an essential role in the numerous challenges the world is facing nowadays, i.e. a rising population to feed, polluted air and soils. Indeed, they are key actors in carbon sequestration, nutrient cycling and provision to plants, plant biodiversity and productivity of natural and agricultural ecosystems (Benner, 2011; Carlile *et al.*, 2001; Hadj-Sahraoui, 2013). Improving our understanding and use of this belowground microorganisms would help limiting chemical inputs in agriculture, allow a qualitative and quantitative optimization of plant production and help remediate degraded soils (Hadj-Sahraoui, 2013).

In the context of research for sustainable development, the production of biofuels and materials from renewable resources is highly required. Therefore, the demand of biomass of all kinds and in particular from micro-algae is growing. World production of aquatic plants reached nearly 27 million tonnes in 2013 (FAO, 2015). Microalgae, in addition to their use in innovative processes (biomethanisation, comethanisation...), produce many molecules/components with diversified functions. The effects of these bio-sourced compounds on plants and mycorrhizal associations are relatively unexplored. However, some near-the-subject studies have revealed stimulatory effects of inter alia microalgal compounds on mycorrhizae opening the door to their potential uses for stimulatory effects on the arbuscular mycorrhizal (AM) fungi. These obligate root symbionts improve mineral nutrition of plants and increase their resistance to abiotic and biotic stresses. Therefore, it would be interesting to know whether micro-algae compounds are able to stimulate mycorrhizal association, thus helping these fungi in reducing the use of agrochemicals (i.e. pesticides or fertilizers) in agricultural production. This master thesis is linked to the MYCO-ALGUE project, contiguous to this approach of seeking durable and natural agricultural innovations.

II. MYCO-ALGUE & ALGO-TECH

The project MYCO-ALGUE is itself part of a broader project, called ALGO-TECH, which aims to set up an integrated platform for the study of the micro-algal sector in order to find valorisation fields for compounds/molecules produced by unicellular algae. In the MYCO-ALGUE project, the goal is to evaluate and select molecules/components derived from microalgae for their bio-stimulation capacities of agricultural and horticultural plants, arbuscular mycorrhizal fungi (to improve their potential for plant stimulation and bio-protection) and for their role in the bio-control of major pathogens in agricultural and horticultural crops.

II. State of art

I. Mycorrhizal fungi

I.I Definition and division

The term mycorrhiza stands for a peculiar type of symbiotic relationship between the mycelium of a fungus and the roots of a plant (Stamets, 2005). Of Greek-Latin origin, the word mycorrhiza means myco (fungus)- (rhiza) root (Fortin *et al.*, 2016). Mycorrhizae can be divided into two major subdivisions: the ectomycorrhizae and the endomycorrhizae. (Peterson *et al.*, 2003). Ectomycorrhizae, are predominant in temperate forest trees. They are characterized by the fact that they do not penetrate the host plant cells. They form a fungal mantle around the plant roots: the Hartig network. Inversely, endomycorrhizae enter the cells of the host plant and form specialized structures, the arbuscules. They are present in an estimate of 80% of plants families belonging both to herbaceous and tree species. A new subdivision is needed to further divide this group of mycorrhizae based on a morphological basis of the nature of intracellular hyphal development. We can distinguish the Arbuscular mycorrhizae (AM), Ericoid mycorrhizae, Arbutoid mycorrhizae, Sebacinoid mycorrhizae, Ectendomycorrhizae and Orchid mycorrhizae (Peterson *et al.*, 2003; Fortin *et al.*, 2016). Ectendomycorrhizae, are characterized, as their name suggests, by both ectomycorrhizal and endomycorrhizal structures (Siddiqui and Pichtel, 2008). The different types of mycorrhizal association are summarized in Table 1.

I.II Arbuscular mycorrhizae

A. Definition and generalities

AM fungi are the most common mycorrhizal type and more than 80 % of the terrestrial plants and almost all cultivated crops associate with them (Hadj-Sahraoui, 2013; Smith and Read, 2008). AM fungi represents only a small group of fungi in the phylum Glomeromycota and omitting a few exceptions, are heterotrophic for carbon (C) and thus obligatorily associate with plants to get their carbon and thus accomplish their life cycle (Peterson *et al.*, 2003; Smith and Read, 2008).

***Table 1**: Different types of mycorrhizae (from Fortin et al., 2016).*

Types of mycorhizzae		Fungi involved	Host plants	Fungal structures	Host structures	Physiological impacts
Ectomycorrhiza		Superior fungi: Basidiomyceta Ascomyceta ~millions sp.	Gymno- and angiosperm trees ~5% of actual sp.	Mycelium mantle, intercellular mycelium, rhizomorphs, sclerotia, ascomata, basidiomata	Cortical hypertrophy, dichotomic or racemose ramifications	Enhanced access to minerals, utilization of organic nitrogen, resistance to pathogens and nematodes, tolerance to acid pH and heavy metals
Ectendomycorrhiza		Deuteromyceta ~few sp.	Pines ~rare	Small mycelial mantle, inter- and intracellular mycelium	Cortical hypertrophy, ramifications	Idem as Ectomycorrhizal fungi
Endo-mycorrhiza	Arbuscular	Microscopic fungi- glomeromyceta ~200 sp.	Bryophytes and vascular plants ~70% actual sp.	Arbuscules and intracellular vesicles, extraradical mycelium and spores	Few changes, yellow color	P, Zn supply, resistance to drought, pathogens, insects, deep metabolitic modifications
	Arbutoid	Basisiomyceta ~few sp.	Ericacea ~rare	Small mycelial mantle, intracellular mycelium, basidiomata	Cortical hypertrophy	Idem as Ectomycorrhizal fungi
	Ericoid	Ascomyceta ~few decades sp.	Ericacea ~5% of actual sp.	Intracellular mycelium, ascomata	Few changes	Idem as Ectomycorrhizal fungi
	Orchid	Basidiomyceta Little-known Sterile mycelium	Orchids ~10% of actual sp.	Intracellular mycelium	Few changes	Often essential to morphogenesis, saprophytic nutrition and protection against pathogens
	Sebacinoid	Piriformospora Basidiomyceta	Varied	Inracellular mycelium	Few changes	Not well-known

Arbuscular mycorrhizae are mycorrhizae forming an arbuscular mycorrhizal symbiosis with plants, which is identified based on intraradical fungal morphology. The term arbuscular refers to arbuscules, which are finely and highly branched hyphal structures thought to be the principal site of nutrient exchange, due to their abundant branches and periarbuscular membranes, that can be found in mycothalli colonized by AM fungi (Akiyama and Hayashi, 2006; Smith and Read, 2008). From the structural diversity of the arbuscular developmental pattern, determined by both plants and fungi, we can distinguish two major classes of associations: The Arum and Paris mycorrhizae, named after the plants in which they were originally observed. The Arum-type regroups most of the cultivated plants and forms intercellular hyphae and arbuscules, while the Paris-type is typical of many trees and forest herbs with their intracellular hyphae, coils and arbusculate coils (Figure 1). The type of mycorrhizal colonization is determined by the host plant and experiments have shown that the same fungus can form both types of colonization, depending on which plant species it is dealing with (Cavagnaro *et al*., 2001; Dickson, 2004). Recent observations in the lab of mycology showed both form within the same root system of one plant (personal communication).

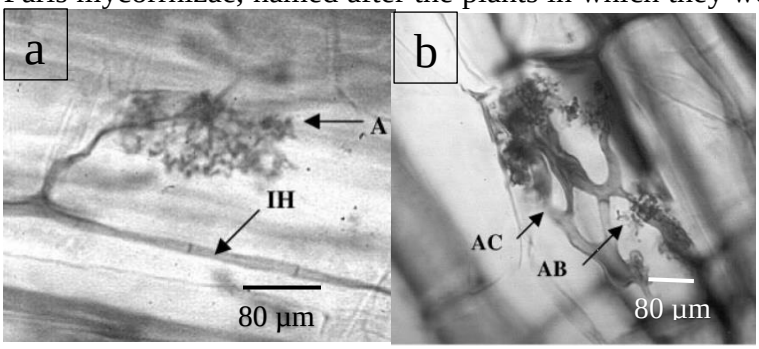


Figure 1*: Typical infection units formed by Glomus intraradices (a) and Gigaspora margarita (b) in association with Lycopersicon esculentum. A, arbuscule; IH, intercellular hyphae; AC, arbusculate coil and AB, arbuscule-like branches (Cavagnaro et al., 2001).*

Arbuscules are not the only characteristic structures of AM fungi. AM fungi also produce intraradical hyphae forming storage vesicles (lipid containing enlarged portions of hyphae) in most genera, and extraradical mycelium with spores (Peterson *et al*., 2003; Smith and Read, 2008).

B. Life cycle

AM fungi are spore producing organisms. Spores are reproductive bodies able of differentiation into a new living organism (Figure 2). They are resistant structures possessing lipid reserves. Therefore they constitute the survival structures of the fungi (Koske and Gemma, 1990). The spores germinate and thanks to the reserve substances they possess, produce presymbiotic mycelia. A diagrammatic representation of the germination process of an AM fungal spore can be found in appendix 1. But spores are not the only propagules capable of germinating. Hyphae from already colonized roots or vesicles can produce presymbiotic hyphae too. In this way, colonization can either arise from a germinating spore, an already colonized root or a vesicle (Peterson *et al*., 2003; Smith and Read, 2008). In order to produce this presymbiotic hypha, propagules do not need a host plant (Ambiwade *et al*., 2012). To increase the likelihood of an encounter between hyphae and a host plant, extensive hyphal elongation and branching should occur. Yet this should happen only if

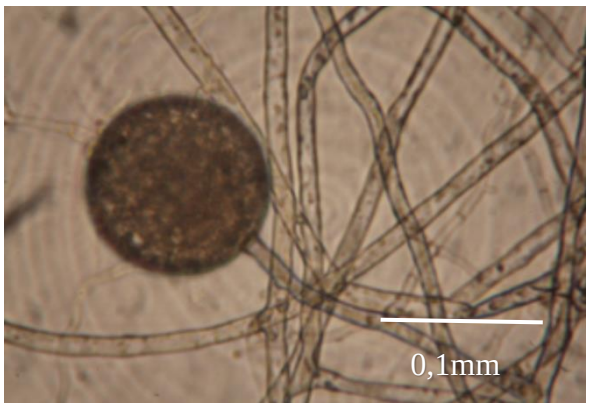


Figure 2*: Spore and hyphae of R. irregularis MUCL 41933.*

the host plant is of reachable extent. According to Akiyama and Hayashi (2006), hyphal branching is the most critical developmental step of the AM fungi's life cycle. Hyphal branching occurs when certain conditions are reunited and ceases before complete drawing of spore reserves in cases of non-proximity of host roots (Akiyama and Hayashi, 2006). In order to avoid drawing of the reserves before reaching a host plant, the pre colonization step of hyphal branching and elongation is initiated by a mutualistic signal exchange between symbionts (Akiyama and Hayashi, 2006). Roots exudate compounds that entail an increase in the metabolic activity of hyphae and hyphal branching (Ambiwade *et al*.,2012; Akiyama and Hayashi, 2006). According to Abubaker et al. (2008), root exudates influence both the membrane activity and the gene expressions of spores during the presymbiotic growth. Akiyama and Hayashi (2006) speak of branching factors (BF's) and define them as lipophilic compounds exudated only by host plants that trigger branching activity of the presymbiotic hyphae. As a response, it has long been assumed that AM fungi would exudate Myc factors that leads to molecular and cellular changes necessary to a successful root colonization. The respiration metabolism would be the first to be stimulated (Akiyama and Hayashi, 2006).

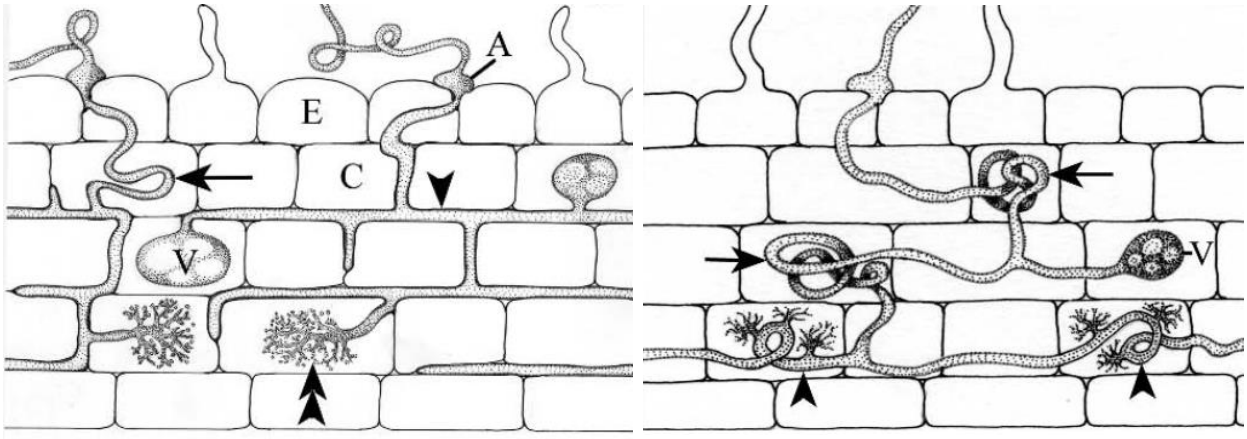


Figure 3*: Diagrammatic representation of root colonization by arbuscular mycorrhizal fungi for the Arum- and Paris-type (Peterson et al., 2003).*

Figure 3 pictures the root colonization of an AM fungi. The left hand side image shows the Arum-type arbuscular mycorrhizal association. Fungal hypha, when it reaches the host plant, differentiates into a specific appressorium (A), the hyphopodium. From here, hyphae penetrate the plant roots through the prepenetration apparatus. The hyphae grow towards the cortex, penetrate the apoplast and move along the root axis to reach the inner root cortical cells. Either in the epidermal cells (E) or in the first layers of the cortical cells (C) coils (arrows) are formed. Inside the cortical cells, successive dichotomous ramifications are going to form arbuscules (double arrowheads) and in some cases, depending on the fungal specie, bulges at the extremities are going to form storage vesicles (Ambiwade *et al.*, 2012; Peterson *et al.*, 2003; Smith and Read, 2008). The Paris-type association is represented in the right hand side image (Figure 3). Root colonization takes the same course as the Arum-type, with some differences. Here, coiling occurs in both the epidermal and cortical cells and in a more extensive way (arrows). This has a consequence that the Paris-type lacks intercellular spaces in the root cortex resulting in a slower intraradical hyphal growth. What's more, the small branches characterizing the arbuscules form on the coils. In the Paris-type, those specific arbusculate structures are called arbusculate coils (arrowheads). For this type of association too, vesicles may or may not form (Peterson *et al.*, 2003). Fungus is not allowed to enter the cytoplasm and periarbuscular membranes are formed by the host to ensure that (Ambiwade *et al.*, 2012).

So, as a summary, an extraradical (ERM) and intraradical mycelium (IRM) are formed. These two mycelia differ morphologically and functionally. The ERM absorbs and transfers nutrients to the host roots while IRM releases nutrients into the apoplast in exchange of carbon. The ERM produces spores resulting from local swellings and the cycle is completed. The reproduction of AM fungi is asexual (Ambiwade *et al.*, 2012; Smith and Read, 2008). Figure 3 is a good capture of this AM life cycle.

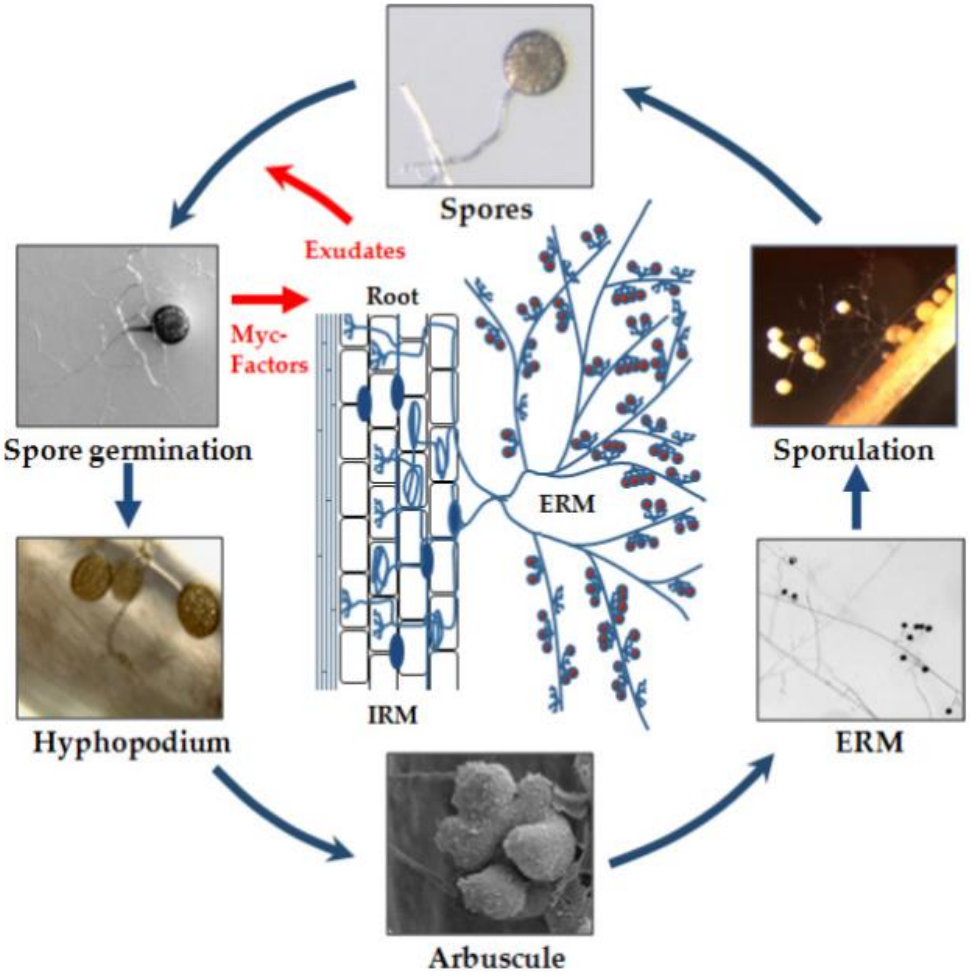


***Figure 4**: Life cycle of an AM fungus and the different steps during AM development (Ambiwade et al., 2012).*

Molecules stimulating hyphal metabolism exudated by plants

The first identified BFs are strigolactones, which is a group of sesquiterpene lactone (Akiayama *et al.*, 2005). It has been discovered that host plants exudate more BFs in phosphate-limited conditions. That is not surprising as BFs trigger hyphal branching and root colonization in view of a mycorrhizal association in which the mycorrhiza can provide phosphorus to the plant.

The BFs are exuded exclusively by host plants, but some other compounds exuded by non-mycorrhizal plants have been reported to stimulate spore germination, hyphal elongation and branching. Some of these compounds are quercetin, myricetin, kaempferol and flavonoids (Akiyama and Hayashi, 2006).

C. Symbiotic relationship

The symbiotic relationship is biotrophic and usually mutualistic, with bidirectional nutrient transfer as the driving force of this interaction. The AM fungus receives carbon from the host plant in exchange for macro and micronutrients as well as water transported by the hyphae to the host root (Smith and Read, 2008). The nutrients exchanged are principally immobile elements such as P, Zn and Cu, but also mobile elements such as S, Ca, K, Fe, Mg, Mn, Cl, Br and N (Tinker, 1984).

Besides nutrient transfer, the interaction can also involve other benefits. For instance, the AM fungus can enhance the drought and disease tolerance of host plants (Smith and Read, 2008). A higher tolerance to drought stress can be due to an increase in water uptake and/or an altered physiology of the plant in order to reduce stress response. A higher resistance of mycorrhizal plants against pathogens can be due to physiological or morphological change. An association with mycorrhizae can also result in an enhanced ability of the plant to produce roots and it can also increase the absorptive surfaces of these ones by altering their morphology, due to the metabolites mycorrhizae can exudate (Linderman, 1988). The mycorrhizal fungus forms a huge underground network and that's why Sutton and Sheppard (1976) bring forward the role of mycorrhizae can have in the soil structure. Mycorrhizal mycelium aggregates soil particles and thus provide a better soil stability. Besides all, this network is the core of connection and communication between plants of the same and different species. Mycelium forms a connection by which messages and nutrients can circulate (Fortin *et al*., 2016).

The mycorrhizosphere, i.e. the zone of soil that is influenced by mycorrhizal roots, represent a local particular environment where microbial activity was demonstrated to be enhanced. Indeed, in presence of the AMF, the physiology and/or morphology of plant roots and thus root exudates is modified, which impacts qualitatively and quantitatively the microbial flora. Linderman (1988) noticed that the myriad of microorganisms found in the mycorrhizosphere is not that different from that found in the rhizosphere. What is different, according to him, are the quantities. These microbes are heterotrophs, but they live and nourish in an environment where the nutrient supply is much increased. They are capable of adapting rapidly to such an improved nutrient supply environment. Mainly sugars, organic acids and amino acids are largely present (Tinker, 1984). The term mycorrhizal microbiome refers to the totality of those microorganisms present in the mycorrhizosphere, from the ones that can be present in the fungal spores, to the ones that form biofilms around the hyphae or that gather around the mycorrhizae (Fortin *et al.*, 2016). As a matter of fact, some of the microorganisms interact with the AM fungi (Linderman, 1988; Fortin *et al*., 2016). For instance, AM fungi closely interact with bacteria to solubilize minerals in order to release nutriments for the host plant. The university of Laval identified AM fungi that appended to apatite in order to favorize the development of bacteria capable of dissolving apatite. By dissolving the mineral, bacteria release phosphorus (P) which is directly reabsorbed by the fungus (Fortin *et al.*, 2016). In the same field of study, Zhang *et al.* (2018) recently discovered a stimulation of the phosphatase activity of a phosphatase solubilizing bacterium in presence of an AM fungi. This one releases fructose, which stimulated the phosphatase genes and the protein secretory system. Subsequently, an enhanced phosphatase activity and thus mineralization of organic phosphorus was observed, increasing the phosphorus uptake by the extraradical mycelium of the AM fungus. Such results demonstrate the wide role played by hyphal and their exudates in the mycorrhizosphere. In this case, fructose doesn't serve only as food, but also as a signal molecule triggering processes in order to maintain a good cooperation between AM fungi and bacteria.

And thus, even though the interaction between AM fungi and microorganisms is far from having revealed all its secrets, several studies have already been made. This does not extend to the interaction between AM fungi and microalgae.

II Algae

II.I Definition and classification

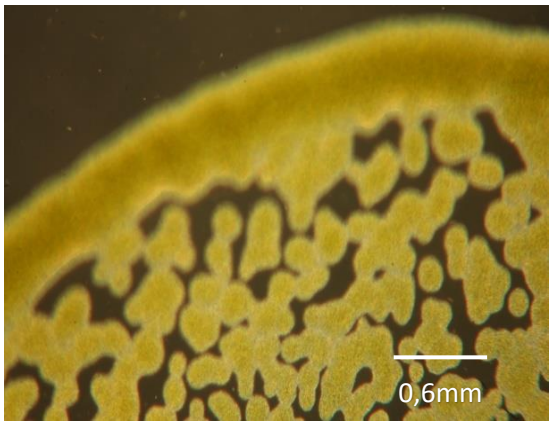


***Figure 5**: S. dimorphus on gelled MSR- medium*

'Algae are a large and diverse group of eukaryotic photosynthetic organisms occurring in almost every habitat. They exhibit a huge morphological diversity, ranging from tiny single cells to huge kelps over 50 meters long (Cocquyt, 2009)'. Together with the land plants, green algae form the green lineage or Viridiplantae. They can be found worldwide, from marine and freshwater ecosystems to terrestrial environments, thus presenting a huge cytological diversity (Cocquyt, 2009; Lalucat *et al.*, 1984). On account of this widespread diversity, algae and in particular green algae are hard to define (Lewis and Mc Court, 2004). In the words of Lewis and McCourt (2004), alga is phylogenetically spoken, a meaningless term. As for green algae (Figure 5), they would define them as 'photosynthetic eukaryotes bearing double membrane-bound plastids containing chlorophyll a and b, accessory pigments found in embryophytes (beta carotene and xanthophylls), and a unique stellate structure linking nine pairs of microtubules in the flagellar base'.

Viridiplantae can be divided in two major groups, that are morphologically distinct; The Chlorophyta and the Streptophyta. Green algae belong mostly to the Chlorophyta (Cocquyt, 2009).

II.II Nutrition

Most algae get their source of energy from light, by performing photosynthesis. They are called autotrophs. Some algae are heterotrophic and get their energy source from organic materials such as proteins, carbohydrates or fats. To do so, the strategies are diverse; Some osmotrophs absorb dissolved substances, while other phagotrophs engulf prey such as bacteria. Some algae are capable of switching from an autotroph way of nutrition to a heterotroph way of nutrition, they are called mixotrophs (Vidyasagar, 2016). Depending on the alga, sugars, acids or alcohols can be added as feed. Acetate, glucose and ethanol are three common substrates used (Richmond, 2003; Lalucat *et al.*, 1984). Heterotrophic nutrition is not only a substitution of the autotrophic nutrition, but it can also happen simultaneously. Hence, the term mixotroph refers to both of these situations (Lalucat *et al*., 1984).

Besides carbon, the two major nutrients a microalga needs are nitrogen and phosphorus. The Redfield ratio of 106: C, 16: N, 1: P gives an estimation of in which concentrations they should be available (Richmond, 2003).

II.III Microalgae

In this master thesis, the studied algae are microscopic algae, called microalgae. They are a diverse group of unicellular photosynthetic micro-organisms, including cells derived from a primary endosymbiotic event (similar to land plants) and cells from subsequent secondary and/or tertiary endosymbiotic events, comprising eukaryotic protists, prokaryotic cyanobacteria and blue-green algae, living in saline or freshwater environments and converting sunlight, water and carbon dioxide to algal biomass (Martinez-Porchas *et al*., 2014; Parker *et al.*, 2008; Ruane *et al.*, 2010).

A. Use

In the last couple of years, microalgae have been more and more used in diversified sectors. Microalgae that are grown autotrophically are used to treat municipal, agricultural, food and industrial effluents as they consume inorganic nutrients such as nitrate and phosphate, which purifies the water. The algal biomass can then be applied to fields as fertilizers or compounds can be extracted. Besides nourishing from nitrate and phosphate, they can also be used for biosorption of heavy metals (Martinez-Porchas et al., 2014; Masojídek and Torzillo, 2008). Microalgae have, the last couple of years, also been used as a source of CO_2 sequestration (Ono and Cuello, 2006).

Even though these organisms are more and more studied and used, in the words of Raja et al., (2008), there is still lots to explore and expect from them as they affirm microalgae are the untapped resource with more than 25,000 species of which only 15 are in use.

B. Hormones

Microalgae contain phytohormones including auxin, abscisic acid, cytokinine, ethylene and gibberellins, that serve as chemical messengers to coordinate cellular activities in higher plants. Although there is much evidence about the positive effects of these hormones in higher plants, their role in microalgae is still unknown. This hormone system has been suggested to be the precursor system of higher plants (Figueroa *et al.*, 2018). The first higher plants that associated with fungi to leave the aquatic environment might have had a similar hormone system than the aquatic plants today. It is also interesting to note that hormones often play an important role in the interactions between organisms.

C. The example of *Chlorella vulgaris*

Definition and classification

Scientific classification: Domain: Eukaryota, Kingdom: Protista, Divison: Chlorophyta, Class: Tre- bouxiophyceae, Order: Chlorellales, Family: Chlorellaceae, Genus: Chlorella, Specie: *Chlorella vulgaris* (Merah *et al*., 2014).

C. vulgaris (*Chlorella vulgaris*) is a green, unicellular, freshwater microalga that exists since 2.5 billion years. This microalga is known for its high protein and lipid content, therefore it has been used primarily as a food source and now for biodiesel production.

Morphology

The microalgal cell is non-flagellated and has a 2 to 10 µm diameter. The cell contains different organelles, similar to those of plants. A scheme of such cell can be found in Figure 6 (Merah *et al.*, 2014).

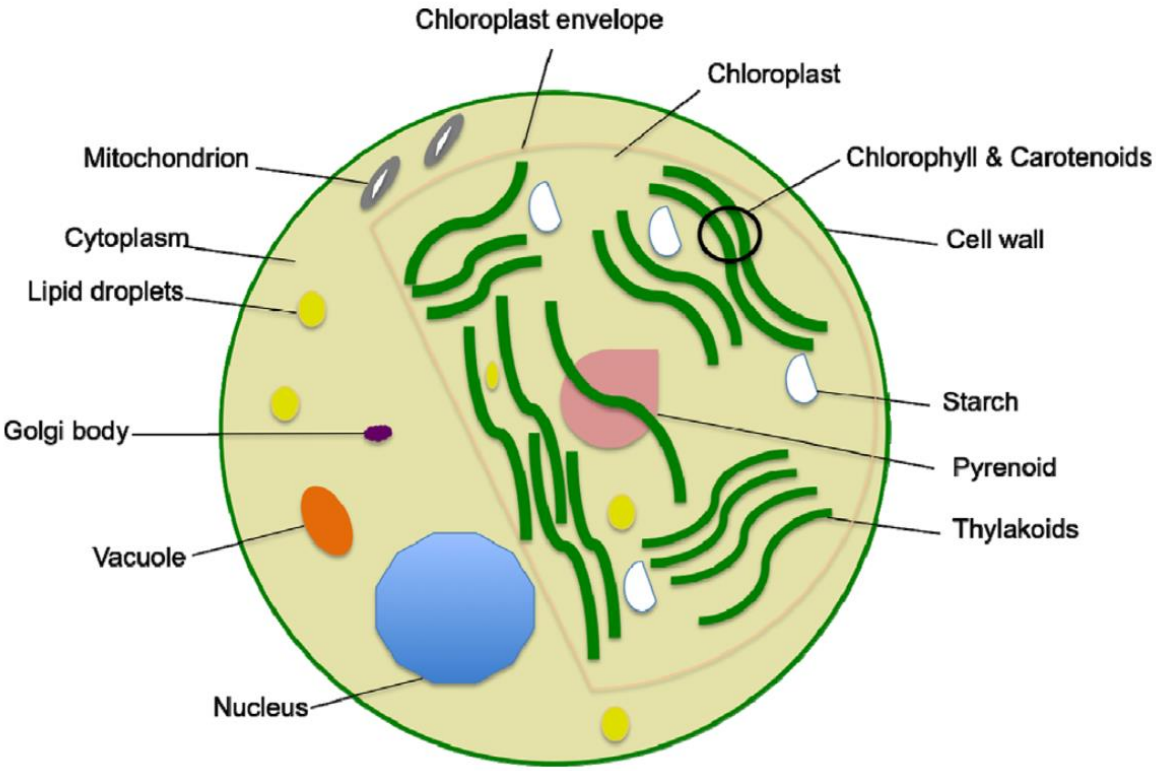


Figure 6: *Schematic ultrastructure of C. vulgaris representing different organelles (Merah et al., 2014.)*

Reproduction

The reproduction of *C. vulgaris* is asexual. In good conditions, it takes 24 hours to the microalgal cell to multiply in 4 distinct cells. This form of asexual reproduction is called autosporulation. When the 4 cells are formed inside the mother cell, the cell wall of the mother cell breaks down, releasing daughter cells and residues of the mother cell that will serve as food for the daughter cells (Merah *et al.*, 2014).

Cultivation

Because of its rapid growth rate and its resistance to bad conditions and invaders, *C. vulgaris* is easy to cultivate. It can be cultivated under heterotrophic, autotrophic or mixotrophic conditions on different organic substrates (Richmond, 2003; Lelucat *et al.*, 1984). Results of growth rates and efficiency of heterotrophic, autotrophic and mixotrophic conditions are mixed. According to Merah *et al*. (2014), heterotrophic and autotrophic growth rates are comparable. Richmond (2003) also affirms that *C. vulgaris* is one of the rare microalgae for which heterotrophic and autotrophic growth rates are comparable. But Degen *et al.* (2011) affirm a

regular photobioreactor provides an autotrophic growth rate of 1.55 g/l/day, while Lian *et al*. (2019) experienced a heterotrophic growth rate of 0.151 g/l/day and a mixotrophic growth rate of 0.254 g/l/day with glucose 1%.
What appears to be unanimous, is that specific growth rate of a mixotrophic culture of *Chlorella* sp. seems to increase with light intensity, in contrast to an autotrophic culture, due to the stimulatory effect of light on the sugar metabolism (Martinez F. and Orus M., 1991; Ogawa and Aiba, 1981; Lee et al.,1996; Lelucat et al. 1984). In mixotrophic conditions and in the words of Lalucat *et al*. (1984), the photosynthetic efficiency of autotrophic defective mutants can be measured as a six-fold of the autotrophic conditions. In this work, the dissipated light and the fraction used for CO_2-fixation during light regime is exploited under both light and sugar regime as an energy source for carbon assimilation. Figure 6 represents specific growth rates of *C. vulgaris* and *S. acutus* of different initial concentrations of glucose, in presence and non-presence of light. As mentioned for *C. vulgaris* before and observed for *S. acutus* here, enhanced growth rate in presence of light is observed. For *C. vulgaris*, a maximum growth rate at an initial concentration of 10 g/l is demonstrated. For *S. acutus*, maximum growth rate is observed at 1 g/l and 1.5 g/l in heterotrophic and mixotrophic conditions. Beyond these concentrations, growth rates conditions are constant, with a growth rate close to zero for heterotrophic conditions. Without presence of light, concentrations higher than 1 g/L are inhibitory.
Besides carbon source and other nutrients, further two important factors influencing the algal growth and chemical composition are temperature and pH (Goswami, 2011).

Use

The cultivation of the genus Chlorella, both in open ponds and photobioreactors, is widespread in the industry because it offers a wide range of uses. It can be used for its biomass, for the synthesis of molecules of interest or in environmental processes such as water treatment. It is also a food traditionally consumed in the East, a medicine or a dietary supplement and it serves as food for mollusc and rotifer larvae in aquaculture. It has also been used in the pharmaceutical field and in the cosmetics sector as a colorant or for another purpose. It is also cultivated for CO_2 abatement (Clement-Larosière, 2014; Merah *et al*., 2104).

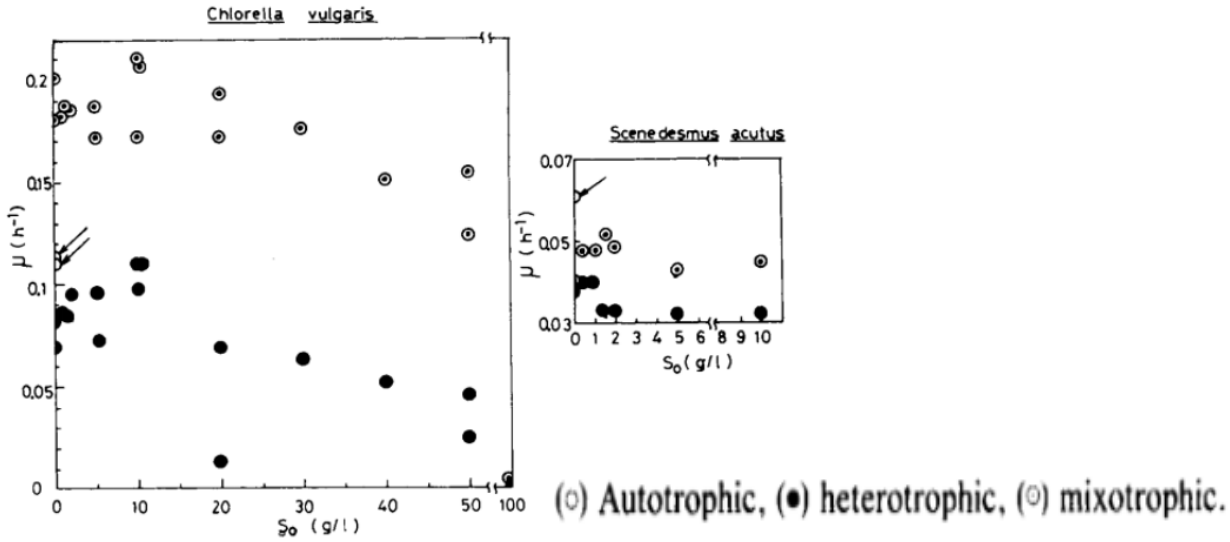


Figure 7*: Specific growth rate vs. initial concentration of glucose, S0 (batch culture)(Ogawa and Aiba, 1981).*

D. The case of *Scenedesmus dimorphus*

Scientific classification: Domain: Eukaryota, Kingdom: Protista, Divison: Chlorophyta, Class: Chlorophyceae, Order: Sphaeropleales, Family: Scenedesmaceae, Genus: *Scenedesmus*, Specie: *Scenedesmus dimorphus* (https://en.wikipedia.org/wiki/Scenedesmus_dimorphus, consulted on 14/05/19)

Scenedesmus dimorphus (S. dimorphus) has been less of a study case than *C. vulgaris* and little research work can be found online. *Scenedesmus* (the genus) can exist in unicells or coenobia of four or eight cells inside a parental mother wall (Lurling, 1999). To replicate, the mother cell enlarges, multiple divisions happen in order to have a multinucleate mother cell. The mother cell then cleaves into uninucleate daughter cells, usually developing as non-motile autospores (Pickett-Heaps and Staehelin, 1975).

No information can be found about the nutrition of *S. dimorphus*, but some other species of *Scenedesmus* had been cultivated mixotrophically. Ogawa and Aiba (1981) cultivated *S. dimorphus* with glucose and Kotzabasis et al. (1999) *S. obliquus* with methanol. Ogawa and Aiba (1981) also experienced a facilitated assimilation of sugars in presence of light for *S. acutus* that has been discussed above. Unlike *C. vulgaris*, Ogawa and Aiba (1981) obtained a higher maximum specific growth rate of *S. acutus* in photosynthetic conditions than in mixotrophic conditions (Table 2).

***Table 2** : Comparison of the maximum specific growth rate of C. vulgaris and S. acutus cultured photosynthetically, heterotrophically and mixotrophically (Ogawa and Aiba, 1981).*

	Maximum specific growth rate (h^{-1})		
	Autotroph	Mixotroph	Heterotroph
C. vulgaris	0.110	0.198 (glucose)	0.098 (glucose)
S. acutus	0.061	0.048 (glucose)	0.040 (glucose)

E. The choice of microalgae

For the ALGO-TECH project, the primary objective of the microalgae is the production of biofuels, molecules with diversified vocations and biopolymers. A first selection of microalgal strains has been made regarding these primary objectives. Then, from a technical point of view, the selected strains had to be freshwater organisms to avoid the use of salt water, which is conducive to corrosion of the installations. Another criterion was the tolerance of the strains to nitrogen hunger, which is necessary at the end of the cultivation period to induce a greater production of molecules of industrial interest. Finally, a more practical aspect was taken into consideration, by selecting microalgae that are easy to grow in the laboratory.

III. Interaction AM fungi-algae

III.I Algal extracts

To date, several studies have shown a wide range of beneficial effects of algal extracts on plants, such as advanced germination, increased plant growth and yield, increased resistance to biotic and abiotic stresses (Beckett and van Staden, 1989; Hankins and Hockey, 1990; Blunden, 1991; Norrie and Keathley, 2006). Although the algae chemical components and their modes of action remain a little unclear, some scientists suggest the modes of action of seaweed extracts to be a synergistic activity (Khan *et al*.,2009). Seaweed extracts are composed of several carbohydrates that are not found in terrestrial plants.

Microalgal exudates have also been proved to be efficient against numerous fungal and bacterial plant pathogens. The effects of microalgal exudates on some beneficial microorganisms associated with plants have also been studied and have provided interesting results (Khan et al., 2009). But little is known about the effects of microalgal extracts have on AM fungi. Up to now, the few studies considering algae and AM fungi have been conducted with macroalgae. For instance, Kuwada *et al.,* (2005) tested different extracts of a brown algae, *Laminaria japonica* on AM fungi. They identified mannitol, carrageenan and other sugar alcohols like xylitol, sorbitol and meso-erythritol that significantly stimulated hyphal growth. Mannitol has also proven to be a stimulator of root colonization and AM fungal plant host growth. Similarly, Ishii *et al.* (2000) observed that oligosaccharides, produced by the enzymatic degradation of alginic acid, mainly from brown or green algae, significantly stimulated hyphal growth and AM fungal elongation.

Apart from these few studies experimenting the effects of macroalgal extracts on hyphal germination and elongation, no further information about experimenting any interaction between microalgae and mycorrhizae can be found (Figure 8).

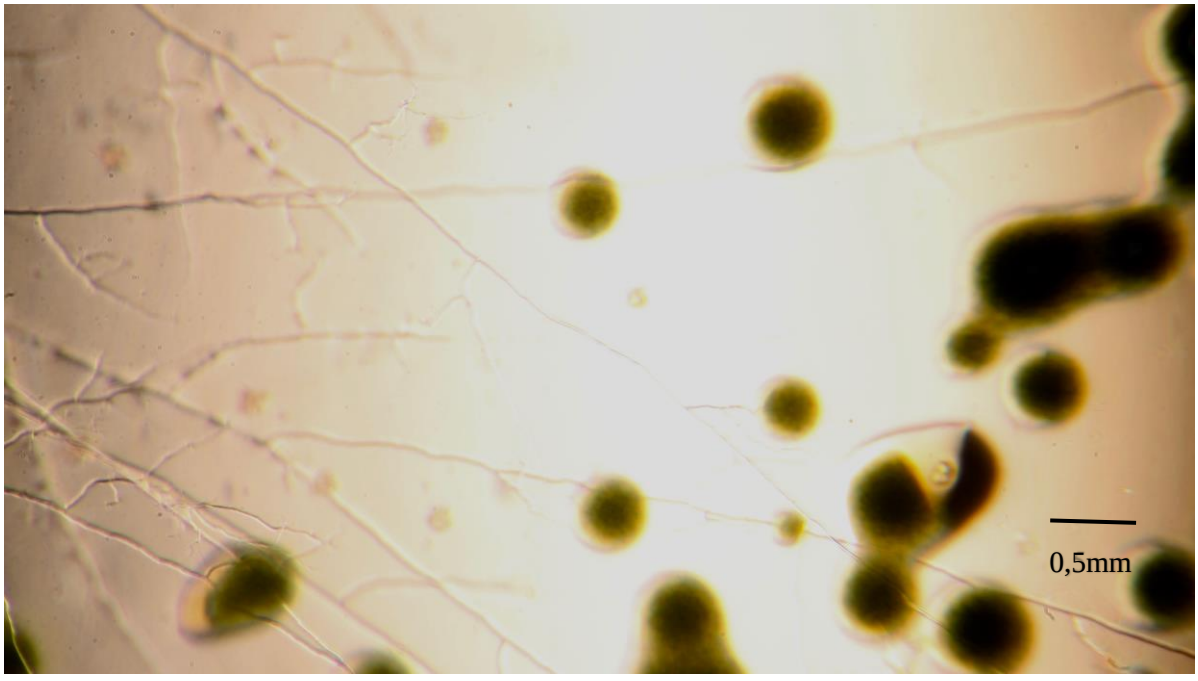


***Figure 8**: Hyphae of R. intraradices MUCL 49410 growing towards S. dimorphus in gelled MSR-medium.*

A special case: the lichen

***Figure 9**:Lichen (https://urlz.fr/9Mos, consulted on 12/5/19).*

The oldest fossil lichen found is 460 million years old. A lichen is an association who ranges from mutualism to control parasitism between a fungus and an alga or a cyanobacterium (Taylor *et al.*, 1995). The alga performs photosynthesis and provides sugars as a source of carbon to the fungus. However, it is suggested that lichens are also able of getting their source of carbon from wood residues regarding to the discovery lichens secrete lignin and cellulose degrading enzymes. This could be of benefit when photosynthesis is not possible, whenever lichens are covered with snow, for example (Wrede *et al.*, 2014). Beside this, the role of the fungus in this mutualistic association is providing protection and procuring water and nutrients (Wrede *et al*., 2014; Fortin *et al*., 2016). Lichens and nowadays ectomycorrhizae too, are able of producing organic acids to dissolve minerals in regard to extract nutrients like phosphorus or potassium.

The success of this beneficial association led to the fact that lichens were the only form of terrestrial plant life on earth for a few millions of years. By entering the rock crevices, as a result of their retraction and expansion related to humification and drought, lichens participated in the fragmentation of rocks, leading to the formation of sand. Moreover, as they are able to use nitrogen from the air, by dying they deposited on the rock the first organic matter rich in minerals and nitrogen. These organic particles mingled with the mineral particles were the basis for a beginning soil formation (Fortin et al., 2016).

IV. Objectives

The main objective of this master thesis was to study the interaction between an AM fungus (*Rhizophagus irregularis* MUCL 41833; *Rhizophagus intraradices* MUCL 49410; *Rhizophagus aggregatum* MUCL 49408 or *Rhizophagus clarus* MUCL 46238) and a micro-alga (*S. dimorphus* or *C. vulgaris*). Numerous studies (Simon *et al.*, 1993; Remy *et al.*, 1994; Taylor *et al.*, 1995; Redecker *et al.*, 2000; Heckman *et al.*, 2001) have reported the role of AM fungi in the colonization of land by plants. The most ancient AM fungi fossil dates back 460 million years ago, which coincides with the colonization of the terrestrial environment by plants (Declerck, 2018). The rocky seats freshly emerged as a result of volcanic action were the first glimpse of land plants could evolve onto and be in competition with one another. It is at this precise moment of evolution that symbiosis played his first act in the development of earthly life, with a natural selection favorizing association rather than competition (Fortin *et al*., 2016). An interaction between aquatic plants and fungi must have taken place and we dare to think such interaction might still exist nowadays. Furthermore, lichens are a proof interaction between fungi and algae still exists because they are the result of a beneficial interaction between fungi and algae or cyanobacteria extending over time (Taylor *et al.*, 1995).

In order to characterize the interaction between AM fungi and microalgae, three main experiments were set up.

In the first one, we aimed to characterize the effects of molecules/components issued from microalgae on the germination and hyphal growth of AMF spores. A positive effect would open doors to a valorisation of microalgae exudates in agriculture. An enhanced mycelial network means an enhanced association with the plant host and thus enhanced benefits of this associations like better growth, resistance to pathogens, etc. (see I.II C. Symbiosis relationship). Soils that has been conventionally tilled for years are soils with very little biological activity and often lacking mycorrhizal mycelial networks. In the context of Walloon conventional agricultural soils, which have been overexploited, farmers are forced to add fertilizers and pesticides. In these soils, communities of mycorrhizae are seriously reduced. In the topsoil, diversity and abundance of spores are significantly lower compared to natural or organic farmed soils (Oehl *et al.*, 2004). An application of microalgal exudates with a view of promoting mycorrhizal association might not be sufficient; An application of microalgal exudates together with mycorrhizal spores could be an attempt of reintroducing mycorrhizae that are not present in the upper soil layers and boost a boom of mycorrhizal associations in the field. A healthy mycorrhized plant could enjoy many advantages over a non-mycorrhized plant with, expectantly, as a result that it doesn't need supply of fertilizers nor pesticides.

The second experiment was set up to evaluate the effectiveness of microalgae filtrates encapsulated with spores in beads on the plant AM fungi symbiosis. The first experiment showed us that microalgae exudates have a stimulating effect on the elongation and branching of hyphae, which are crucial elements for a good colonization of the plant and AM fungus-plant association. The third experience takes this first experiment a step further; By encapsulating microalgae exudates with mycorrhizae spores in beads that are applied in the vicinity of roots, we would like to measure the number of hyphae that have successfully emerged from the beads and their elongation. This experience is closer to the more applied objective of enhancing the value of exudates in agriculture. These beads could be applied to the field as biofertilizer/biopesticide.

With the third experiment, a more fundamental question is tried to be answered. Many scientists evoke that microalgae can be found in soils: 'Anywhere sunlight and water co-occur' (Sandesh Suresh et al., 2019). Microalgae can be found on the surface layers of soils, where light passes through and they can realize photosynthesis. Thus, these microalgae that perform photosynthesis are, in a natural context, not likely to be in contact with mycorrhizae, as mycorrhizae don't like sunlight. What is not known, is if heterotrophic microalgae can be found in deeper layers of the soil where no light passes through.

So, it is not known if microalgae and mycorrhizae can be found in the vicinity of each other and if they interact together. However, this is a question a good number of scientists have looked into. Smith, chief business office of Heliae Development LLC, which is a company that tested many different microalgae having positive effects on agricultural plants, affirms that microalgae are entirely part of the soil ecosystem: 'A lot of what we're doing is actually restoring what is already present in the soil. A lot of the scientists we work with think this is a critical component of the whole plant ecosystem below the soil line.' The company developed a product called PhycoTerra®, which is a whole natural, pasteurized algae-based cell, that is meant for both organic and conventional soils. In their words, applying algae in the soil can increase yields, crop qualities and strengthen soils (Cavanaugh, 2016; https://heliaeglobal.com/industries/agriculture/, consulted on 25/5/19). Although no scientific articles have appeared on the underground heterotrophic microalgae community, people working in this field are convinced there is one.

This experiment pushes the question a little bit further by setting the assumption that it is the mycorrhizae that provides sugars to the microalgae. One can imagine that the microalgae offer something else in exchange, such as exudates that triggers mycorrhizal association, as is experienced in the first part of this thesis work. The objective is thus to observe if microalgae can prosper in a whole dark environment with no source of sugars, except the one that can potentially be given by mycorrhizae.

III. Materials and methods

I. Biological Material

I.I Plant roots

- ***Daucus carota L.***

Genetically modified root organ cultures (ROC) of carrot was used. These roots have undergone a hormonal modification that enables them to grow without the help of a shoot through sub-cultivation. Root organ cultures of carrot are adequate to study the development of AM fungi under *in vitro* culture conditions (Fortin *et al*, 2002):

- The risk of contamination in lower than with *in vitro* cultures of plants. Indeed, Petri plates containing ROC do not need to be opened regularly for feeding, contrarily to plants *in vitro* cultures.

- They allow an easy observation of root, fungal and algal growth.

- They are kept under controlled conditions (in the dark). In this master thesis, some experiments were conducted in the dark and it would have been an additional issue to deal with plants that need to perform photosynthesis.

- Gain of space for storage in comparison with cultures of plants in pots.

I.II Arbuscular mycorrhizal fungi

The four selected strains were provided by the Glomeromycota *In vitro* Collection (GINCO) in the BCCM/MUCL facilities.

- ***Rhizophagus irregularis (Blaszkowski, Wubet, Renker & Buscot) C. Walker & A. Schüßler***

Figure 10*: R. irregularis spore and hyphae*

Strain number: MUCL 41833
Conditions for growth: Modified Strullu-Romand medium (Declerck et al., 1998)
Origin: Canary Island

Spore size: 70-165 µm in diameter.
Spore color: Almost hyaline to yellow brown.
Spore, subtending hyphal and hyphal wall: Spore, subtending hyphal and hyphal wall contain three layers, formed sequentially.
Occlusion: Recurved septum (from inner lamina of spore wall layer), plug, or open.

R. irregularis is a frequently used strain under *in vitro* conditions and particularly in the mycology laboratory of UCLouvain (Voets et al., 2009). Its genome has been fully sequenced (Beaudet *et al*., 2018).

- ***Rhizophagus intraradices (N.C. Schenck & G.S. Sm.) C. Walker & Schueβler***

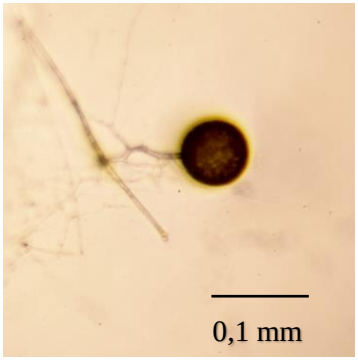


Figure 11: *R. intraradices spore and hyphae*

Strain number: MUCL 49410
Conditions for growth: Modified Strullu-Romand medium (Declerck et al., 1998)
Origin: America, United States, Florida, Orlando, Clermont-Minneola

Spore size: 40-140µm in diameter.
Spore color: White, pale cream to yellow brown, sometimes with a green tint.
Spore, subtending hyphal and hyphal wall: Subtending hypha and spore wall contain three layers that are formed sequentially. Spore wall contains 3 layers.
Occlusion: Innermost sublayer(s) of the third layer of the spore wall which do not extend into the subtending hypha and occasionally a plug partitions spore from hyphal contents.

- ***Rhizophagus aggregatum***

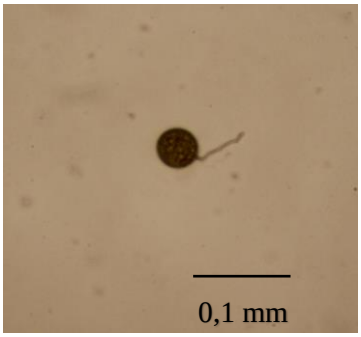


Figure 12*: R. aggregatum spore and hyphae*

Strain number: MUCL 49408
Conditions for growth: Modified Strullu-Romand medium (Declerck et al., 1998)

Spore size: 40-120µm in diameter.
Spore color: Hyaline to pale yellow in color.
Spore, subtending hyphal and hyphal wall: Spore wall contains 1 to 2 layers. The subtending hyphae usually is single but occasionally double. Hyphal wall consists of one or both layers of the spore wall.
Occlusion: Usually open, sometimes a thin septum, thickening of the spore wall, or a cytoplasmic plug.

- ***Rhizophagus clarus (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler***

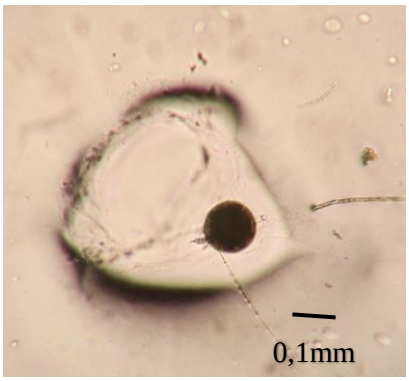


***Figure 13**: R. clarus spore and hyphae*

Strain number: MUCL 46238
Conditions for growth: Modified Strullu-Romand medium (Declerck et al., 1998)
Origin: America, Cuba, Pinar del Rio, La Palma

Spore size: 100-260µm in diameter.
Spore color: A continuum from white to yellow-brown, with many pale yellow to pale yellow brown.
Spore, subtending hyphal and hyphal wall: The spores and hyphal wall contain three layers which may all be adherent but the third layer often separates in mature spores and are formed consecutively as the spore wall differentiates. The subtending hyphal wall has also three layers, with the first layer thinning to invisibility within 5 µm of the spore surface.
Occlusion: Innermost sublayer of the third layer of the spore wall bridges the pore (often it is so thin that the pore appears to be open), sometimes the spore wall thickens and almost occludes the pore and a plug is formed with rare frequency.
(http://fungi.invam.wvu.edu/the-fungi/species-descriptions.html, consulted on 24/07/19)

I.III Microalgae

- ***Chlorella vulgaris & Scenedesmus dimorphus***

The strains were provided by Dr. Anne-Lise Hantson (Umons).

Cell wall of the daughter cell *C. vulgaris* increases until it reaches 17 to 21 nm and a microfibrillar layer is formed, composed of glucosamine, which gives the cell wall its rigidity. After maturation, cell wall thickness and composition change according to growth and external conditions.
No information was found on the wall structure of *S. dimorphus.*

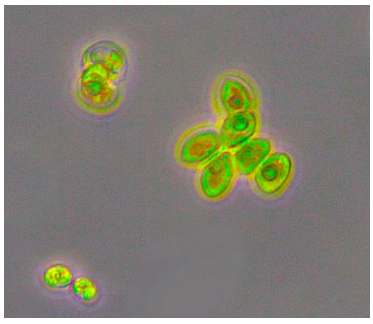


***Figure 14**: C. vulgaris (https://www.algotherm.com/algue/chlorella-vulgaris/, consulted on 11/07/19)*

II. Methodology

II.I *In vitro* culture methods

A. *In vitro* root organ culture of *Daucus carota*

The first step to set up a culture is to prepare the growth medium. Transformed *Daucus carota* (DC) roots were cultivated on the Modified Strullu-Romand (MSR+) medium containing sucrose (10 g/L) and vitamins (Declerck *et al.*, 1998). The pH was adjusted to 5.5. The MSR+ medium as well as the MSR- medium (without sucrose and vitamins) was solidified with 3 g/L of Phytagel. Details about the composition and preparation of MSR- and MSR+ can be found in appendix 2.1.

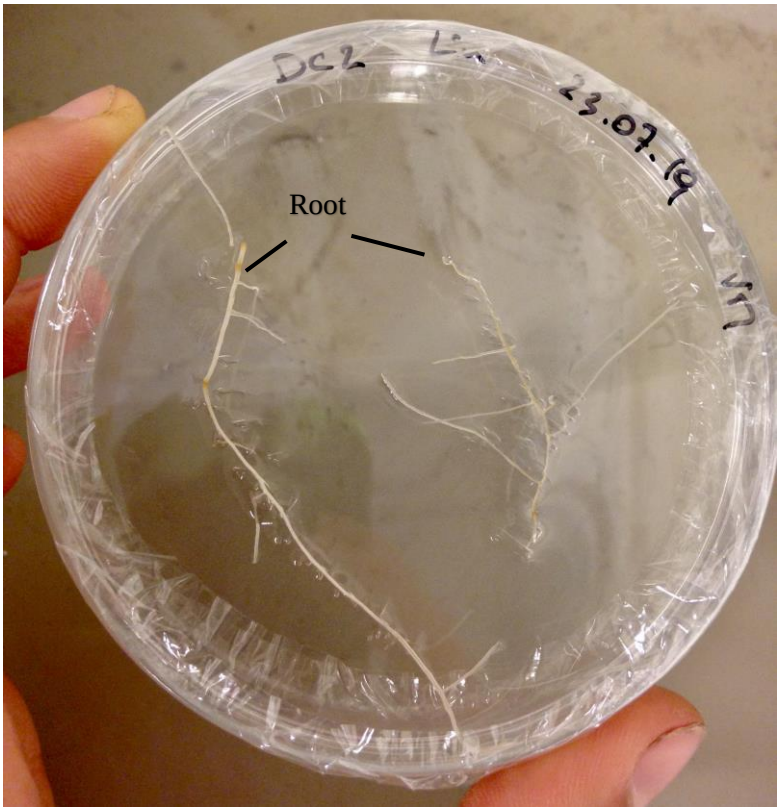


***Figure 15**: Petri dish with Daucus carota roots on MSR+ medium*

After autoclaving (121°C during 15 min), the MSR medium was poured into 90 mm mono-compartmented Petri dishes, under laminar hood. After solidification of the MSR medium, two carrot roots of 3 to 4 cm long and containing lateral apexes, were selected from the stock culture and placed on the MSR medium with their apexes gently pushed into the solidified medium. The Petri dishes were closed with a cellophane film and are incubated at 27°C, in the dark. They were turned upside down because the modified DC have a negative geotropism. New cultures were initiated once a month, in order to have modified carrot roots stock. Figure 17 represents a 2 weeks old root organ culture.

B. *In vitro* culture of mycorrhizae

The AM fungi were kept on ROC (Root organ culture) of carrot in bi-compartmented Petri dishes (St-Arnaud *et al.*, 1996; Figure 18). MSR- and MSR+ media were both prepared in Erlenmeyer flasks. After autoclaving, the media were poured into 90 mm bi-compartmented Petri dishes until solidification. 25 ml of each medium wase poured into the two compartments of the bi-compartmented Petri dishes. The MSR+ medium was poured in the root compartment (RC) and MSR- medium in the hyphal compartment (HC). Each manipulation was made under sterile conditions. When the media were solidified, roots were placed in the RC compartment, as explained in the *in vitro* culture of DC roots above. Finally, a dissolved gel inoculum was used to inoculate the root: about 50 spores of one of the above-mentioned fungi were deposited close to the root as uniformly as possible. The Petri dishes were then closed with cellophane film, turned upside down and incubated at 27°C, in the dark. Roots crossing the plastic barrier from RC to HC were regularly trimmed with forceps and scalpels. New cultures were made monthly in order to have an AM fungi stock to inoculate whenever is needed (Caron *et al.*, 1996).

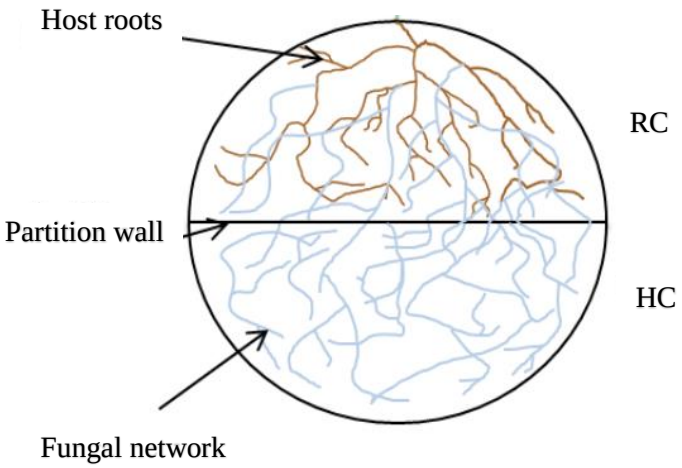


***Figure 16**: bi-compartmentalized Petri dish with a root compartment containing roots and a fungal network and a hyphal compartment containing a fungal network.*

C. *In vitro* culture of microalgae

In 3N-BBM medium

- Cultures of microalgae in Erlenmeyer flask

Microalgae were cultured in 1L Erlenmeyer flasks at 25°C in the daily light, under agitation (110 rpm). Active cultures were maintained every 3 weeks by transferring ¼ of the old culture medium into ¾ of new sterile (autoclave 121°C during 15 min) 3N-BBM medium (pH 7). Details about the composition and preparation of 3N-BBM can be found in appendix 2.2. The plateau phase is reached at 3 g/L of dry biomass.

- Culture of microalgae in Petri dishes

In order to initiate Petri dish cultures, microalgae cells were collected in an actively growing 3N-BBM liquid culture medium of the microalgae and resuspended 9 times (dilution 1/10), followed by 3 cascade dilutions from 10 to 10 until 1/10000, in 3N-BBM. For each dilution, 100 µL of suspension was dispersed on a 90 mm Petri dishes of 3N-BBM containing agar (6 g/L) with 2 replicates for each concentration and strain. Dispersion on the medium surface was made with a modified Pasteur pipette. The Petri dishes were then stored in the phytotron

(16h/8h day/night, 21/19°C, relative humidity of 70%, average photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}) and used as back-up for a period of up to 6 months minimum. After two weeks to up to six months, the Petri dishes were used to initiate new cultures under sterile conditions. The microalgal colonies were scraped with a flame sterilized scalpel and introduced into a liquid MSR medium in Erlenmeyer flask.

When the culture is contaminated, going through a Petri dish culture and scraping only a pure microalgal colony in order to reintroduce it into a liquid medium, is a way to make a new contaminant-free cultures. What is more, initiating a Petri dish culture allows to detect if the liquid culture is contaminated. On gelled medium, it is much easier to detect the development of contaminations as compared to a liquid medium culture. To ensure that the liquid culture is pure, a Petri dish culture can be launched and if no contamination has developed after 1 to 2 weeks, it is assumed that the liquid culture is pure.

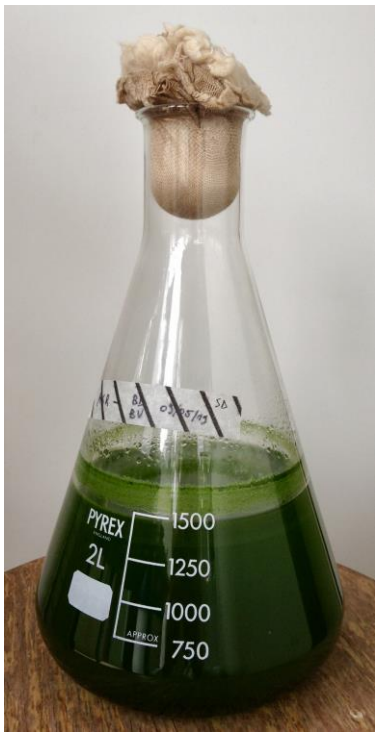


***Figure 18**: Liquid MSR- culture of S. dimorphus*

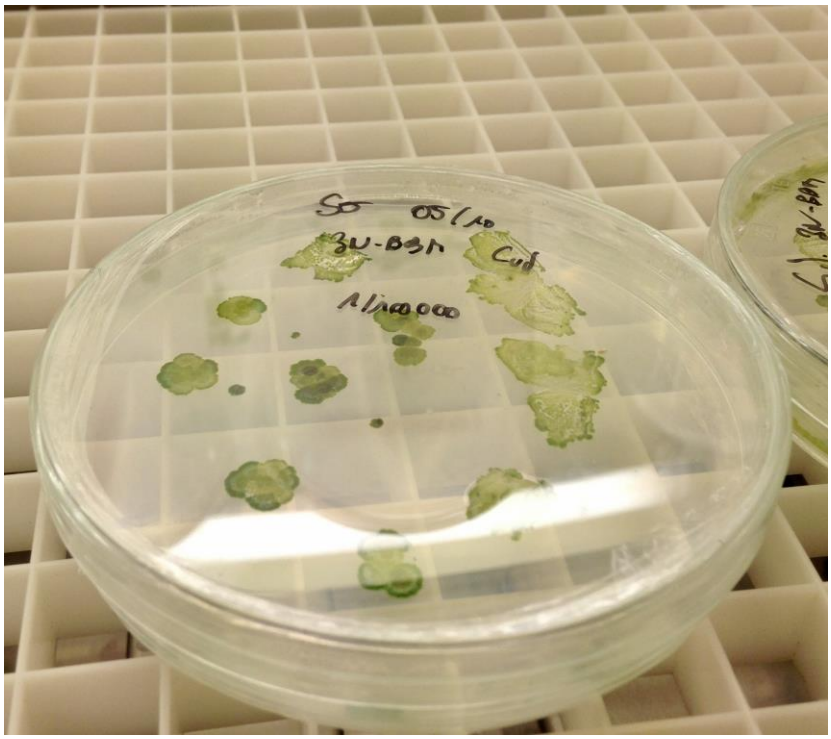


***Figure 17**: Petri dish culture of S. dimorphus on 3N-BBM medium*

In MSR(+ and -) medium

To transfer from a 3N-BBM culture medium to the MSR (+ and -) medium, cells from the liquid 3N-BBM solution were resuspended, diluted and dispersed on a 90 mm Petri dish of MSR (+ and -) containing Phytagel (3g/L). The Petri dishes were stored in the phytotron (16h/8h day/night, 21/19°C, relative humidity of 70%, average photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}). It is then possible to initiate liquid MSR (+ and -) cultures from the MSR (+ and -) Petri dishes as explained for 3N-BBM cultures above.

Filtration of microalgae supernatants

In order to obtain microalgal exudates free of microalgae, supernatants were filtered with a vacuum pump with a series of filters of decreasing porosity to prevent clogging of the latter. To cross the filter more easily, supernatant was aliquoted in Falcon tubes of 50 ml and centrifuged at 3000 rpm at 23 °C for 10 minutes prior to the filtration.
The porosity used was as follows:

- 2.7 µm (Whatman®)
- 0.8 µm(Pall©)
- 0.45 µm (Pall©)
- 0.2 µm (Pall©)

Filters were changed every 10 ml because bigger volumes clog the latter and inhibit the filtration process. After filtering to the minimum porosity, it was imperative to autoclave 0.2 µM filters and the entire filtration system, including the bottle, to perform a final filtration under sterile conditions under a laminar flow hood. The supernatant was then aliquoted in Falcon ® tubes and stored at 4°C (not in the freezer, to avoid degradation of the molecules of interest).

II.II Experimental set-up

A. Impact of microalgal exudates on the germination of spores, hyphal branching and elongation of germinating hyphae

Effects of microalgae exudates from 3N-BBM medium

- Implementation

Supernatant of *S. dimorphus* cultivated on 3N-BBM medium was collected at the Umons laboratory in Duran bottles. The supplied supernatant had previously been centrifuged, in order to have only a small number of cells left in suspension. It was important to process the samples quickly enough to prevent the development of contaminations or multiplication of microalgae. This is why it was imperative to keep the supernatant at 4°C and to carry out filtration as quickly as possible. Supernatant was filtered as explained above.

In order to evaluate the effect of molecules exudated in the supernatant of 3N-BBM medium, different concentrations of supernatants were tested. For this, MSR- Petri dishes containing four different concentrations of microalgae exudates were prepared. The concentrations were the followings: 25, 2.5, 0.25 and 0% of microalgae exudates.

MSR- medium containing Phytagel (3 g/L) was prepared and autoclaved as explained above. Four empty sterilized Erlenmeyer flasks were disposed under a laminar flow hood. Seventy-five % (of the final volume) of MSR- medium was transferred in every Erlenmeyer flask, together with 25 % (of the final volume) of exudates in the first Erlenmeyer flask, 2.5 % of exudates and 22.5 % of sterilized water in the second one, 0.25 % of exudates and 24.75 % of sterilized water and 25 % of sterilized water in the third and fourth Erlenmeyer flasks

respectively (see Figure 21 and Table 3). Once the four Erlenmeyer flasks of distinct concentrations were ready to be used, the contents were poured into Petri dishes. Ten ml medium was poured into 50 mm Petri dishes. Ten replicates were considered per working solution (WS) of microalgae exudates.

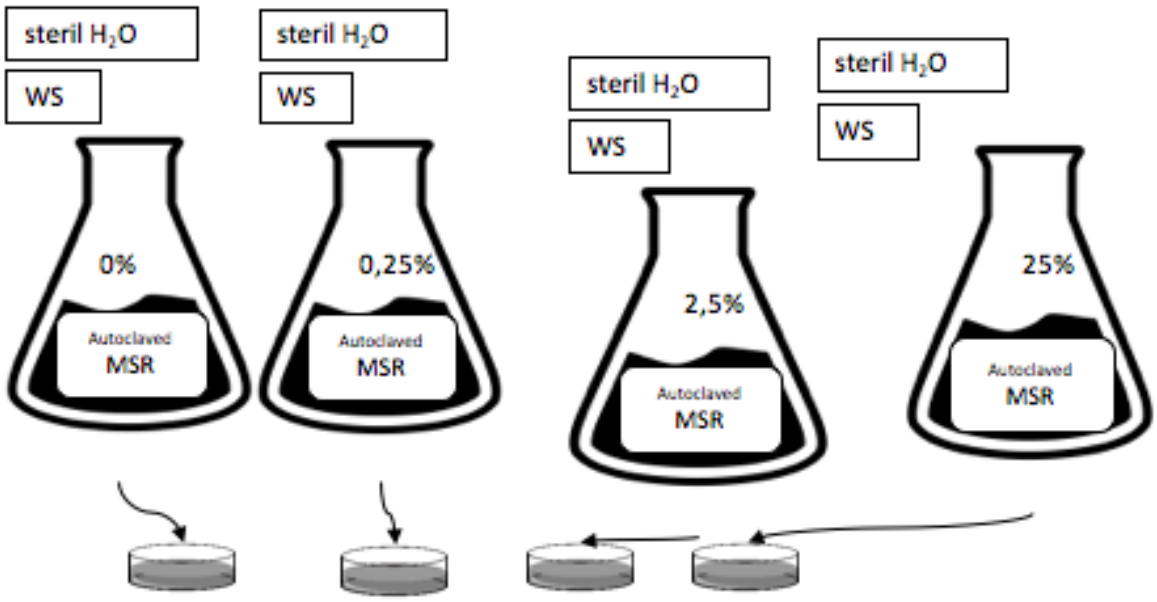


Figure 19: *Preparation of Petri dishes to investigate the impact of microalgae exudates on AM fungi spore germination, hyphal branching and elongation. The MSR medium contained 0, 0.25, 2.5 and 25% supernatants of microalgae.*

Table 3*:Proportion of working solution (WS) of Micro-algae supernatant.*

	0%	0,25%	2,50%	25%
Number of replicates	10	10	10	10
Volume of medium	600 mL	600 mL	600 mL	600 mL
Sterile MSR-	450 mL	450 mL	450 mL	450 mL
WS	0 mL	1,5 mL	15 mL	150 mL
H2O	150 mL	148,5 mL	135 mL	0 mL

The experiments were conducted with the 4 strains of AM fungi (*R. irregularis* MUCL 41833, *R. intraradices* MUCL 49410, *R. aggregatum* MUCL 49408 and *R. clarus* MUCL 46238). After four months of *in vitro* culture in bi-compartmented Petri dishes, an active fungal mycelium developed in the HC. A piece of medium (about 4 cm^2) from the HC containing spores of one strain was sampled and placed into an emptied, sterile Petri dish. By mean of a flame sterilized scalpel, the medium was cut through during 5 minutes in order to cut the hyphae and release single spores. With a syringe and sterile Acrodisc® filters (0.22 µm, Pall), the citrate buffer (about 4 ml) was applied in order to dissolve the MSR medium. Details about citrate buffer composition and preparation can be found in appendix 3. The isolated spores were then transferred to a Petri dish containing sterile water. Spores were finally selected under a binocular microscope (magnification of 2.5) and transferred to the prepared Petri dishes

containing 25 % - 2.5 % - 0.25 % - 0% of microalgal exudates. A good selected spore is a non-broken spore with a small subtending hypha. Twenty replicates were considered per concentration. Petri dishes were closed with cellophane film and incubated at 27°C in the dark.

The quality of the spores used was first tested by incubating the spores on MSR- medium and assessing their germination potential. Six spores were tested and the culture was considered acceptable when 4 out of the 6 spores germinated within the two weeks.

- Monitoring

Pictures were taken of each spore the day they were placed into the Petri dishes or the day after. To take the pictures, a camera with a special end cap was fixed onto the microscope. Pictures were taken at 40 to 100 X magnification. The Petri dishes were observed under the microscope every two days. After germination of a spore, pictures of the hyphae were taken every two days for a period of 8 days. According to Tommerup (1983), germination is defined as the production of a germ-tube up to 5 µm in length. Further elongation is described as hyphal growth. After three weeks, the spores that did not germinate were considered as non-germinating spores.

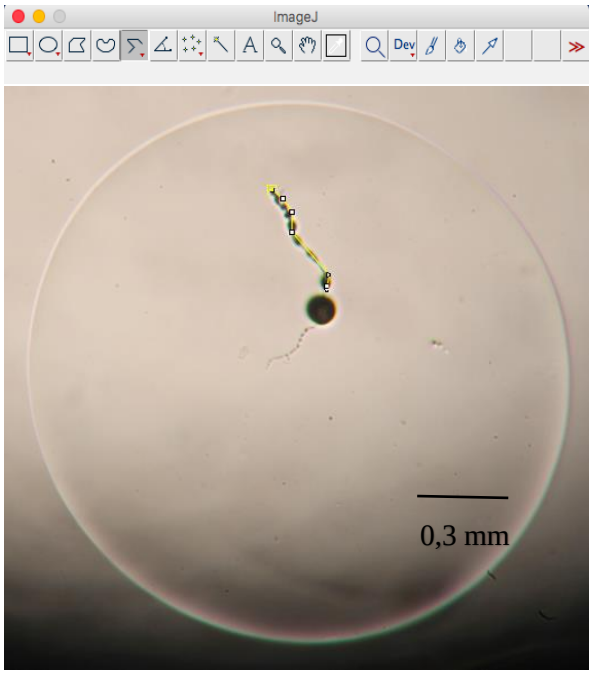


***Figure 20**: Calculation of hyphal length of a R. intraradices MUCL 49410 spore with ImageJ program.*

Four variables were measured. The two first relate to spore germination. The first variable is the rate of spore germination for the same treatment of the same strain, at the end if the three weeks. The second measured variable is the day at which the spore germinated, with day 0 = the day the spore was applicated in the Petri dish. The two other variables relate to hyphal growth of a germinated spore. The first one is the measure of the hyphal elongation. To this extend, pictures were downloaded and hyphal elongation was measured by mean of the software imageJ. The last measure is the number of hyphal branches, that has been counted on the pictures. Hyphal elongation and branching were reported to a daily growth and branching in order to compare results with other experiments.

As the implementation and the monitoring of these experiments are time consuming and the germinating tests were not always conclusive, experiments on the four strains were realised at different time periods.

Effects of microalgae exudates from MSR+ and - medium

As the culture medium of the supernatant was 3N-BBM, a medium containing nutrients at higher concentrations than the MSR medium, we hypothesized that the 3N-BBM medium could

directly have an impact on the germination and growth of AM fungi. It was then decided to use MSR medium.

- Implementation

In order to conduct this experiment, microalgae were cultured and filtrated in MSR(+ and -) medium first. For this experience, supernatants of *S. dimorphus* and *C. vulgaris* cultured in autotrophic and heterotrophic conditions were used.

Four cultures of *S. dimorphus* and *C. vulgaris* were established under light and dark conditions.

- For the heterotrophic cultures, 1 g/L of glucose was added in the MSR medium. The erlens were packed in aluminum foil and placed in an agitator under daylight (ambient termperature, 110 rpm).
- For the autotrophic cultures, no glucose nor vitamines were added in the MSR medium. The cultures were placed in an agitator in the daylight (ambient room temperature, 110 rpm).

After 45 days, a small volume per culture was sampled and the concentration of microalgae was measured with a Fuchs-Rosenthal cells. Dilutions were made in order to have the same number of microalgae in all cultures and thusly the same number of molecules/compounds. As the liquid MSR culture was made by scrapping some colonies on the MSR Petri dishes, the number of introduced *C. vulgaris* and *S. dimorphus* cells per culture was not the same at the beginning. For *C. vulgaris*, an equal development has been observed: counting test revealed the same concentration of microalgae after 45 days in the heterotrophic and autotrophic culture. For *S. dimorphus* on the other hand, microalgae had developed almost twice as much in the autotrophic culture than in the heterotrophic one. The best grow was observed for *S. dimorphus* cultivated in the dark, followed by *C. vulgaris* cultivated in the dark and in the light and finally *S. dimorphus* cultivated in the light.

The cultures were then filtrated, as explained above. The other steps were similar to the experiment with 3N-BBM exudates, except that MSR + (glucose 1g/L + vitamins) was poured instead of MSR- in the Petri dishes. Regarding the AM fungal strains used, the germination tests were realized and all experiments initiated with the first strain that passed the germination test, which was *R. irregularis* MUCL 41833.

- Monitoring

The monitoring of this experiment was similar to the experiment with 3N-BBM exudates.

B. Effects of microalgal exudates encapsulated with mycorrhizal spores on the germination of spores, hyphal branching and elongation of germinating hyphae

By encapsulating microalgae exudates with mycorrhizal spores in beads that are applied in the vicinity of roots, we would like to measure the number of hyphae that have successfully emerged from the beads, the elongation of these hyphae and finally the root colonization.

- Implementation

    - The preparation of beads (taken up and modified from Lalaymia, 2013)

The encapsulation of *R. irregularis* MUCL 41833 spores with microalgae supernatant was carried out as follows: Four-month *in vitro* cultures of *R. irregularis* MUCL 41833 containing spores and mycorrhizal carrot roots were milled twice with sterile water in a sterile mixer for about 30 s at 20.000 rpm. The mixture was then distributed in several 50 mL sterile Falcon® tubes. Each mixture was filtered through a sterile nylon mesh (30 µm Ø). Spores accumulate on the meshes and were dried before being temporarily stored in a sterile Petri dish. The spores on the nylon meshes were then recovered by dipping them in a 4% (w/v) solution of sterilized sodium alginate.The alginate mixture obtained was homogenized and then distributed in 3 Petri dishes. To make 3 different types of beads, each alginate solution was mixed with 3 different solutions. Thus, three types of beads were formed with:

- Control beads without supernatant, where an equal volume of a liquid MSR- was added to the alginate mixture.
- Beads with 100% supernatant, where an equal volume of raw and filtered supernatant from a culture of *S. dimorphus* in MSR- medium was added.
- Beads with 10% supernatant, where an equal volume of *S. dimorphus* supernatant diluted to 10% in an MSR- liquid medium was added.

The different alginate mixtures were homogenized. Before doing the polymerization of the beads, several sterile pipette cones were prepared with tips cut with a chisel. This step facilitates the formation of drops when pipetting the alginate solution. Three drops are placed on a slide and observed with a binocular magnifying glass to check the average spore quantity per drop. The polymerization of the beads took place in sterile $CaCl_2$ (0.1 M) solution. To form a serie of beads, 800 µL was sampled from the alginate mixture with a pipette, from which the cone was cut. This volume was dropped drop by drop into the $CaCl_2$ solution under agitation. When a drop of the alginate mixture came into contact with $CaCl_2$, the bead began to polymerize. The polymerization lasts about 15 minutes. Once the polymerization was complete, the beads were recovered by draining them with a previously sterilized tea ball. Each batch of beads was then rinsed with sterile water and stored in separate Petri dishes.

    - The preparation of Petri dishes with beads and carrot roots

A total of 30 mono-compartmented 90 mm Petri dishes were poured with MSR+ medium (10 Petri dishes for each treatment). In each Petri dish, five beads were placed with a sterile forceps in the culture media just medium fusion. Several pieces of carrots were cut and placed around the beads, about 1 cm away from the beads (Figure 23). The Petri dishes were then closed with parafilm and cellophane.

- Monitoring

The germination of the AMF from the beads (expressed as a percentage) was measured after 4 and 30 days. The length of hyphae emerging from the beads after 4 days were measured (Figure 24). Three types of lengths were considered: the average length of the hyphae, the total length of the hyphae per bead, the total length of the hyphae per Petri dish. Pictures of all hyphae were taken and lengths were measured with the software ImageJ.

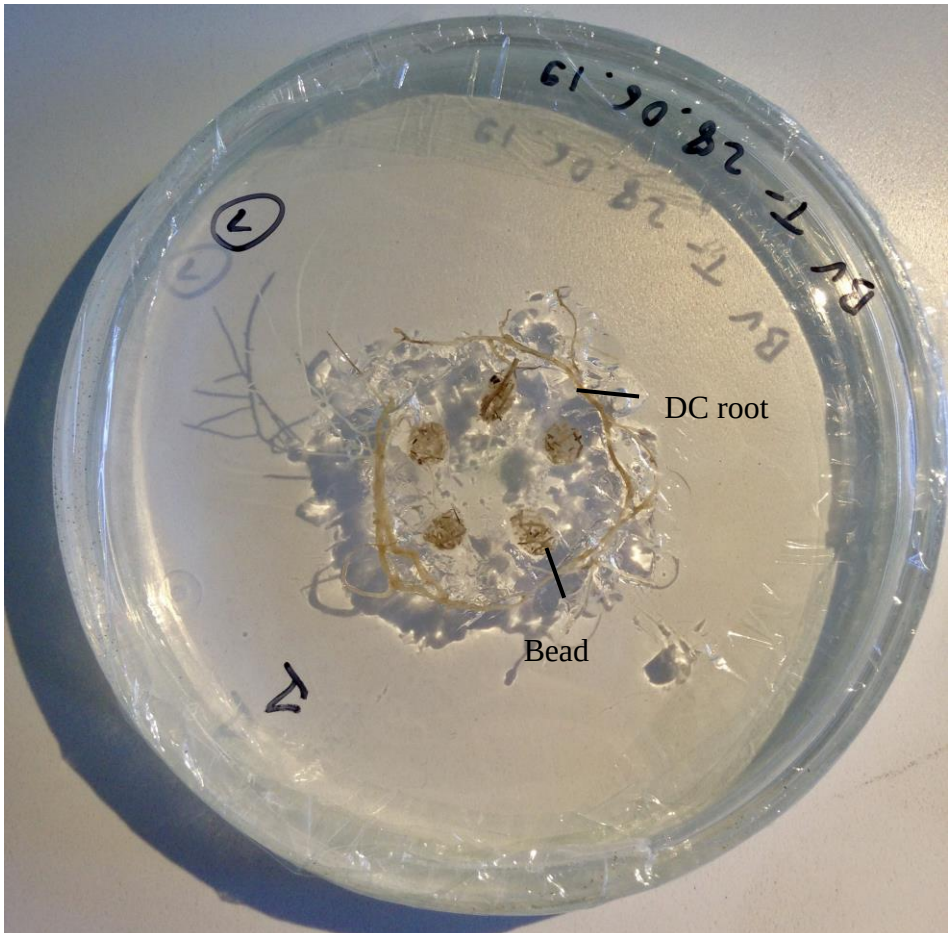


***Figure 21**: Petri dish with MSR+medium, Control beads containing R. irregularis spores and no exudates, and DC roots.*

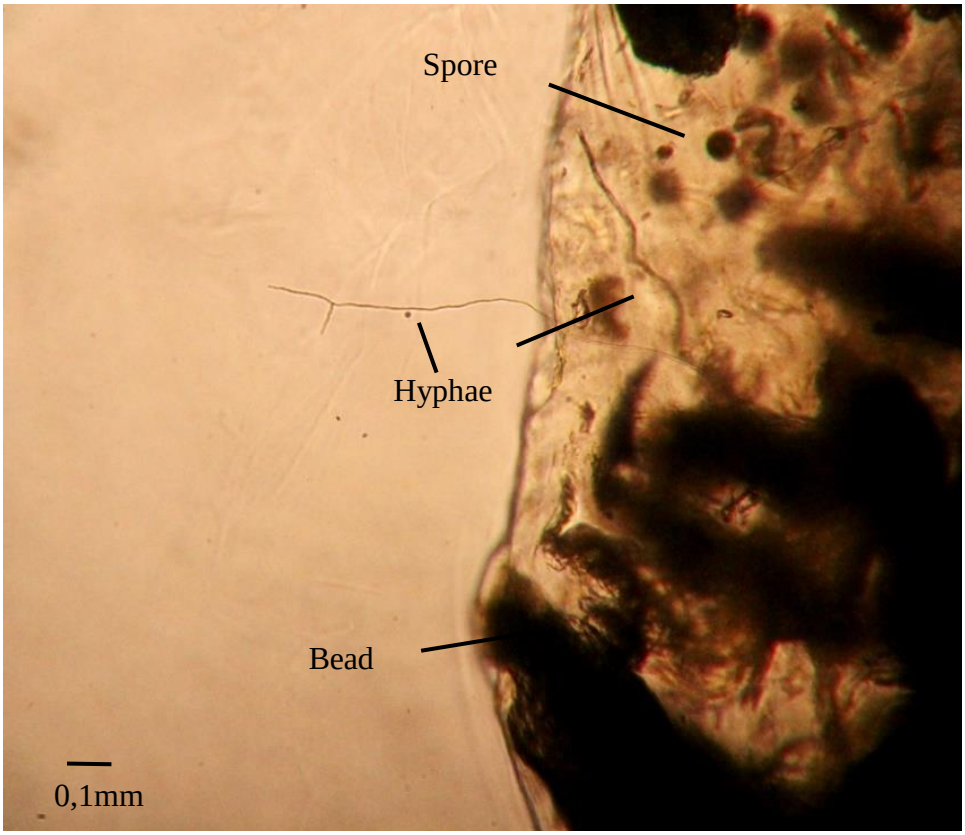


***Figure 22**: Bead containing spores and hyphae (and 10% exudates) on MSR+ medium Petri dish*

C. Effect of AM fungal hyphae exudates on the microalgae growth

The main objective of these experiments was to determine if microalgae are able to develop under dark conditions with no source of sugars, except the one potentially provided by hyphal exudation.

Preliminary assays

All the assays were realized with *S. dimorphus*. The tests did not always follow each other in time. Some were launched in parallel or before obtaining the results of the previous ones.

- Determination of adequate dilution to stimulate microalgae growth

In order to see which dilution was needed to obtain only a few colonies of microalgae on Petri dishes, cells were collected in an active liquid culture and resuspended in 1:9 (1/10), followed by 3 cascade dilutions from 10 in 10 to 1/10.000 in 3N-BBM. For each dilution, 100 µL of suspension was dispersed on a 90 mm Petri dishes of 3N-BBM (as explained for the microalgal culture in the section above). This dilution was needed to facilitate the counting of the colonies and to follow their development.

- Effects of streptomycin on *S. dimorphus* growth

In an attend to inhibit photosynthesis and to detect how microalgae develop in heterotrophic conditions (color, growth ,…), streptomycin was added to the medium. For *C. vulgaris*, Streptomycin was found to be an inhibitor of the synthesis of the D1 protein and perhaps other coded chloroplasts proteins that are part of the electron transport chain, essential in photosynthesis (Perales-Vela *et al.*, 2016). Concentrations used were based on the scientific literature (Perales-Vela *et al*., 2016). Four concentrations of 0, 0.12, 0.24 and 2.4 mg/L were added to gelled MSR- medium.

- Growth of *S. dimorphus* in heterotrophic and mixotrophic cultures

a) Liquid cultures

As we did not know if *S. dimorphus* could grow heterotrophically, which is crucial information for this experiment, a liquid MSR+ (10 g/L sucrose) culture was started and placed in the dark. Additionally, two liquid MSR + cultures with 1 g/L and 10 g/L of glucose were initiated and placed in the dark. Glucose seemed to be a better option as *S. acutus* and *C. vulgaris* had both been successfully cultivated with glucose in heterotrophic conditions before (Richmond, 2004) and it has been demonstrated that mycorrhizae exudate glucose (Zhang *et al*., 2018). For *S. acutus*, initial concentrations of glucose up to 1 g/L revealed to be appropriate for heterotrophic nutrition; above this concentration and certainly with 10 g/L, development was inhibited (Ogawa and Aiba, 1981). As no information was available for *S. dimorphus*, the two concentrations were tested.

b) First Petri dishes cultures

In parallel of the liquid cultures, cultures on Petri dishes were initiated. Two replicates of each of the following combinations of Petri dishes were prepared: 25 ml of MSR-, MSR+S (10 g/L sucrose), MSR+G1 (1 g/L glucose) or MSR+G10 (10 g/L glucose) with or without streptomycin

(2.4 mg/L) were added in mono-compartmented Petri dishes. Twice 10 µL of dilutions 1/10 000 and 1/100 000 of *S. dimorphus* were applied and the Petri dishes were placed in the phytotron (16h/8h day/night, 21/19°C, relative humidity of 70%, average photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}) in the light or in the dark (covered with aluminium foil).

c) Second Petri dishes cultures

The experiment was replicated, except that streptomycin and glucose at 10 g/L concentration were kept aside. Streptomycin did not seem to provide the attended results, as for glucose 10 g/L concentration, it seemed to inhibit algal growth. So this concentration was indeed not wanted for heterotrophic culture of *S. dimorphus*. For this experiment, the microalgae were first purified, by mean of an intermediate Petri dish culture, as explained in section C. *In vitro* culture of microalgae. Triplicates of mono-compartmented Petri dishes were initiated of two dilutions 1/10 000 and 1/1000 of the combination of MSR, MSR+S, MSR+G1 in the light and in the dark. A bigger volume (100 µL spots) was also applied on the surface medium.

- Interaction between AM fungal hyphae and microalgae in heterotrophic conditions on solid medium.

Two to three months old bi-compartmented Petri dishes containing AM fungal cultures of *R. irregularis* MUCL 41833 and *R. aggregatum* MUCL 49408 grown on MSR+ medium in the RC and MSR- medium in the HC were selected to produce actively growing hyphae on the surface medium of the HC. Four droplets of 5 µL of 1/100 and 1/1000 dilution of *S. dimorphus* were added on the surface medium next to the hyphae. One Petri dish was placed in the light, the other one in the dark. This was also done for two other Petri dishes that contained no hyphae in the HC, as control. For this test, it was important to note that some roots had crossed the plastic barrier and grown in the HC, even if droplets of microalgae did not touch the roots around.

Final experiments

- Implementation

The final experiment was composed of three treatments of which two are controls. Figure 25 shows the experimental set-up.

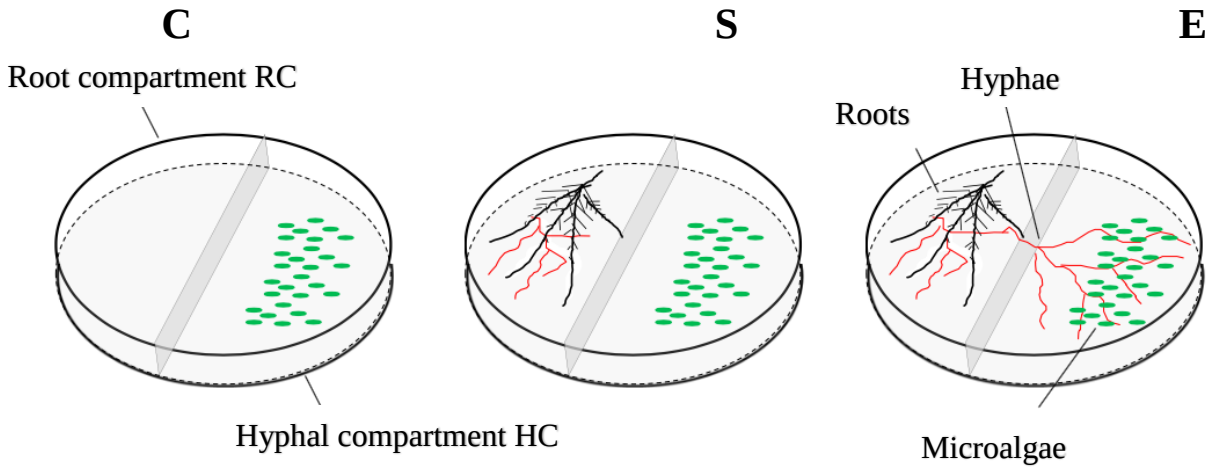


***Figure 23** : Impact of AM fungal hyphae exudates on the growth of microalgae cells. Three treatments are considered (C), Petri dishes with no roots and AM fungus in the root or hyphal compartment (RC or HC, respectively), (S) petri dishes with roots and AF fungus in the RC only, and (E) Petri dishes with roots and AM fungus in the RC and AM fungal hyphae extending in the HC (adapted from Zhang et al., 2018).*

In order to refer easily to the different treatments, letters were assigned to each of them. In the first treatment, no roots nor AM fungi were present in the root compartment. This treatment was named the Control (C) treatment. In the second treatment, roots and AM fungi were present in the root compartment (RC), but did not cross the plastic barrier separating RC from HC. This treatment was named the S treatment. In the last treatment, roots and AM fungi hyphae were present in the root compartment and hyphae crossed over the plastic barrier to the HC. This treatment was named the E treatment. In all Petri dishes, microalgae were only deposited in the HC. The RC and HC were composed of MSR+ (MSR with 10 g/L sucrose) and MSR- medium respectively. The MSR- medium was either liquid (exp.1) or gelled with Phytagel (exp. 2 and 3).

To make the E (and S) Petri dishes, 105 cultures of the 4 strains of mycorrhizae (*R. irregularis* MUCL 41833, *R. intraradices* MUCL 49408, *R. aggregatum* MUCL 49410 and *R. clarus* MUCL 46238) were initiated, with timing differences, as explained in section II.I B. *In vitro* culture of mycorrhizae, except that the MSR- compartment was not fully filled. Three to four mL MSR- medium was added in a slope from top to bottom of the plastic barrier. Once the hyphae of enough Petri dishes have crossed the plastic barrier, about 6 mL MSR- can be poured in the whole compartment. In view of monitoring growth in a gelled MSR- medium, the reason of adding the MSR- medium in a slope first permits to reduce the differences of hyphal growth and expansion in the hyphal compartment. In view of monitoring growth in a liquid MSR- medium in the microalgal compartment, pouring a slope allows to see when enough Petri dishes are ready to be used. The slope is then removed and replaced by the liquid medium.

Twenty Petri dishes without roots and mycorrhizae were prepared at the same time as the ones with the AM fungal strains, in order to make the C Petri dishes. For the S treatment, the Petri dishes prepared for the E treatment in which a poor to almost no hyphal growth and expansion in the RC would occur, would be used. After 3 months of incubation at 27°C in the dark, the first Petri dishes were ready for use.

Experiment with liquid hyphal compartment

- Further implementation

Fourteen and sixteen Petri dishes inoculated with *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 with well-developed hyphae in the HC, 14 and 16 other Petri dishes inoculated with 41833 and 4910 that produced few hyphae in the HC and 12 Petri dishes with no roots nor mycorrhizae in the HC were used to initiate the experiment. Gelled MSR- slopes were removed with a sterile scalpel and replaced by 10 mL of liquid MSR medium. The Petri dishes were further incubated as previously described. Every three days, the Petri dishes were observed for hyphal growth in the liquid medium. The number of replicates per treatment differed due to contaminations. In the C treatment, 8 Petri dishes for both the strains *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 were used. In the E treatment, 8 and 10 Petri dishes respectively for the strains *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 were used. Finally, in the S treatment, 9 and 9 Petri dishes for the strains *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 were used.

Before applying the microalgae onto the HC, Petri dish cultures were started from the liquid culture, in order to ensure that the liquid culture was not contaminated, as explained under the section II.I C. *In vitro* culture of microalgae. After 25 days, liquid MSR- media of the HC was collected and replaced by 10 ml of liquid MSR medium containing 10^{-9} cells/l of *C. vulgaris*. The initial concentration of microalgae applied was based on the one used by Clement-Larosière (2012) in her experiment of observing *C. vulgaris* growth rate under different CO_2 concentrations. The Petri dishes were wrapped into aluminum foil and incubated in the dark.

- Monitoring

Several observations were made:

a) Microscopic observation

Petri dishes were observed to see if differences in microalgal growths were perceptible, or if any interesting interaction could be noticed. Pictures were taken under the microscope (magnification 40x, 100x, 400x).

b) Counting cells

This method consists in measuring the concentration of *C. vulgaris* by a Fuchs-Rosenthal counting cell. On day 7, 9, 11 and 13, 20 µl of the liquid culture was homogenized with a sterile tip in the HC and then sampled. For each sample, cells in 18 squares were counted. Their mean was calculated and was used to find the concentration of that sample. From the number of cells counted in one square, the microalgae concentration of the sample was deduced with next formula:

cell concentration = [total counted cells / number of squares] x80.

The sampled volume was replaced by the same volume of pure MSR- liquid medium. To avoid falsified results by letting a strand of light pass through that could activate photosynthesis of algae, the sampling took place in a dark room.

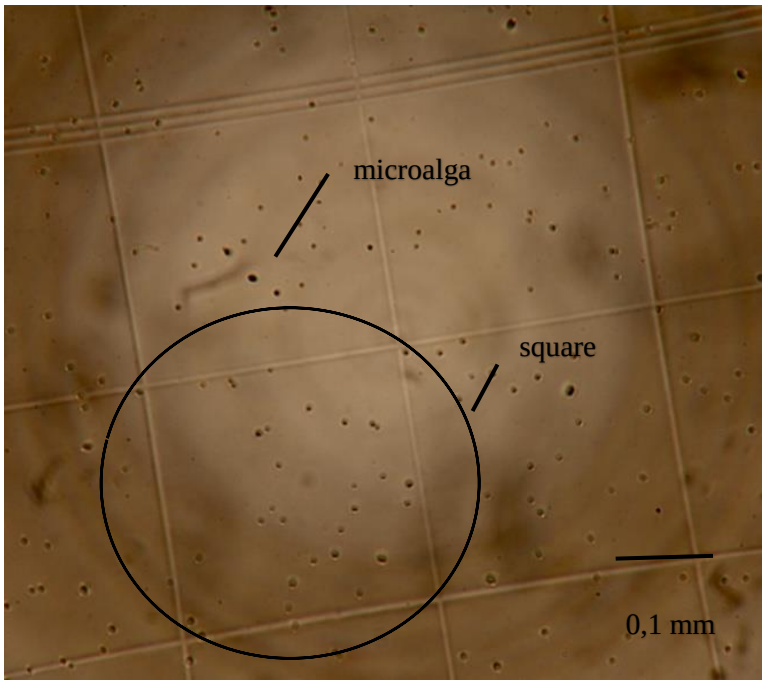


***Figure 24**: Microscopic picture of C. vulgaris cell on a Fuchs-Rosenthal cell.*

c) OD measurement

After the last sampling on day 13, a part of the volume was used to make OD measurements. OD measurements were realized with a DU 800 Spectrophotometer. Absorbance of the sample was measured with single wavelengths readings. Absorbance (A) can be explained by the following formula (Durfee, 1998) :

$$A = \log(\frac{P0}{P})$$

P0: Light given off

P: Light being received

The greenest looking sample was selected and a wavelength scan was first realized in order to detect which wavelength had the highest rate of absorbance. The graph showed a peak absorbance at 680 nm. In a previous experiment outside this study, algal biomass of *C. vulgaris* and *S. acutus* was been measured with a fixed wavelength of 550 nm (Ogawa and Aiba, 1981). Fixed wavelengths readings were taken with those two wavelengths.

To convert absorbance into algal biomass, the next formula was applied (Ogawa and Aiba, 1981):

$$X\ [g/L] = OD_{550}(0.33 \pm 0.01).$$

In addition, algal mass concentrations were adjusted to the remaining volume, by mean of the next formula:

$$X_{adjusted}\ [g/L] = (\ X[g/L]V[mL]\ ) / 10[mL].$$

d) Adjusting to the remaining volume

After the last uptake on day 13, the entire volume of microalgal culture was sampled and measured with a serological pipet. It was assumed that the final volume of all the HC would not be heterogeneous after 13 days, for three reasons: The evaporation and the hyphal absorption, the sampling and the liquid that is inadvertently spilled in the other compartment or onto the lid of the Petri dish. Since the volumes would not be identical in all Petri dishes at the end of the experiment, the final concentrations were adjusted to the remaining volume. Furthermore, in order to reduce the decrease in volume per sample, the sampled volume of MSR liquid was re-added. This results in a small dilution that can be calculated and therefore adjusted.

$V = 20\ \mu L \times 3 = 60\ \mu L$ (V=sampled volume before last sampling)

$C_{na} = X/10\ 000$ cells/ μL (C_{na}=non-adjusted concentration, X=number of microalgae)

$C_a = X/(10\ 000\text{-}60)$ cells/μL (C_a=adjusted concentration)

$\Rightarrow C_{na} / C_a = 10\ 000/(10\ 000\text{-}60)$

$\Rightarrow C_{na} / C_a = 1{,}006$

$\Rightarrow C_a = C_{na} / 1{,}006$

Thus the final concentration should be divided by 1.006 in order to eliminate the effect of sampling. What is not considered here is that a dilution of microalgae potentially recedes them into their development cycle and might be at the base of an enhanced growth. A diluted concentration can bring the microalgae in another phase of their development, for example the exponential growth phase again if the plateau phase was reached. Anyhow, as it has been demonstrated the dilution is minim and will not cause any problem of that kind.

Experiment 1 with gelled hyphal compartment

- Further implementation

Five and 6 well-colonized Petri dishes and 5 and 6 poorly-colonized Petri dishes inoculated with *R. clarus* MUCL 49408 and *R. intraradices* MUCL 49410 respectively, as well as 5 Petri dishes with no roots nor mycorrhizae were chosen for conducting this experiment. Five ml of

gelled MSR- medium was poured in the continuum of the MSR slope in the HC. The Petri dishes were incubated as above. Every three days, the growth of mycelium in the HC was checked. After 21 days, the HC of most of the Petri dishes were well-colonized and the experiment was launched. Two spots of 5 µl of dilution 1/1000 of both *S. dimorphus* and *C. vulgaris* were applied in the vicinity of hyphae.

- Monitoring

a) Microscopic observation

At day 1, Petri dishes were observed under the microscope and pictures of the microalgal spots were taken. The Petri dishes were then wrapped in aluminum foil and replaced in the incubation chamber.

After 14 days of incubation in the dark, the Petri dishes were observed under the microscope. Pictures of the same microalgal spots that had been photographed before were taken and all the spots were observed (Figure 27).

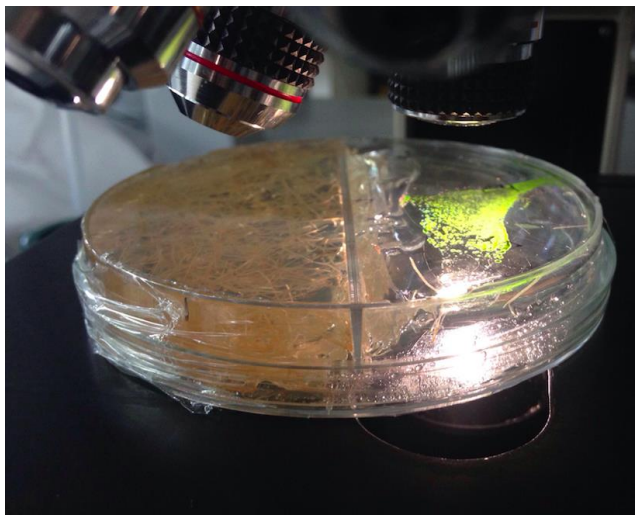


***Figure 25**: Microscopic observation of microalgae c in the bi-compartmented Petri dish.*

b) Phosphorus tests

In order to compare the phosphorus concentrations between the compartments with and without hyphae, phosphorus measurements were performed. They were performed by Anne Iserantant of the Mineral and Organic Chemical Analysis Laboratory at the Earth and Life Institute of Environmental Sciences. Phosphorus was measured after filtration using an inductively coupled plasma spectrometer (ICAP 6500 duo view) from Thermo Scientific.

Experiment 2 with gelled hyphal compartment

The previous experiment was repeated with changing certain aspects of the protocol in an attempt to eliminate certain hypotheses that could have contributed to the unattended results of that experiment.

In the first gelled experience, 3 weeks had passed between the moment the medium was poured in the hyphal compartment and the moment the microalgae had been applied on it. Mycorrhizae could have sampled most of the mineral ions in this compartment. For this experiment, microalgae were applied right after the solidficatoin of the MSR- medium to decrease the chances the hyphae have 'prélevés' all the minerals from the medium.

Furthermore, as mycorrhizae tend to dive into the medium and that microalgae are applied to the surface, microalgae were often not in direct contact with mycorrhizae.
In order to maximize the chances of encounter between hyphae and microalgae, the MSR- medium film was poured as thinly as possible (about 5 mL).

Finally, a slightly more diluted initial concentration was chosen and the microalgae were applied in a fine line at the boundary between the slope of MSR filled with hyphae and the freshly poured new solid medium.

- Further implementation

For this experiment, 5 of both well- and poorly-colonized Petri dishes inoculated with *R. intraradices* MUCL 49410, as well as 5 Petri dishes with no roots nor mycorrhizae were chosen. The tiniest possible layer (about 5 mL) of gelled MSR- medium was poured in the prolongation of the MSR slope. Two lines of 100 µl of *S. dimorphus* and *C. vulgaris* of dilution 1/3000 were applied in both halves of the HC. Petri dishes were photographed under the microscope (magnification of 40, 100 and 400) before being wrapped in aluminum foil and placed back to incubation to obtain 5 plates of treatment E, 4 plates of treatment C, 4 plates of treatment S.

***Figure 26**: camera set-up on the microscope*

- Monitoring

After 14 days of incubation in the dark, the Petri dishes were observed under the microscope and pictures of the same photographed spots were taken (Figure 28).

II.III Statistical analysis

In order to detect significant differences between increasing concentrations of 3N-BBM microalgal exudates and between different strains (2 factors) on germination day, daily hyphal growth and daily hyphal ramification, an ANOVA1 was realized between the controls of the different experiments. If the controls were not significantly different, a non-parametric Kruskal-Wallis test was used. It analyses the effect of a variable on the measured data. If $p<0.05$, a Tukey test is performed. These two tests were realized on the R program.

To detect significant differences in germination day, daily hyphal growth and daily hyphal ramification between increasing concentrations of MSR (+ and -) exudates and the two strains (*C. vulgaris* and *S. dimorphus*) cultivated in dark on one hand and *C. vulgaris* cultivated in the light or in the dark on the other hand (2 factors), the same tests (Kruskal-Wallis test and Tukey test on the R program) were used.

Additionally for the first experiment testing the effects of microalgal 3N-BBM exudates on hyphal elongation of germinated spores, two other statistical analyses were conducted. A non-parametric correlation test followed by a Kruskal-Wallis test was realised in order to ensure that the length of subtending hypha did not interfere with the results. A one-factor ANOVA (95% confidence interval), conducted with the SAS program, was supplemented by a Tukey test, realized on the R program to compare the means respectively, to explain the distribution of hyphal elongation obtained on the basis of differences caused by supernatant concentration. To do this, the homogeneity of variances was checked by a Levene test. If these variances were not homogeneous, then the data were transformed by different functions (logarithmic, inverse or square root arcsine). This was conducted for different data of *R. intraradices* MUCL 49410 over time.

ANOVA1s (SAS program) followed by Tukey tests (R program) were also conducted on the OD measurements and the microalgal concentrations of the counting tests of the experiments testing the effect of AM fungal hyphae exudates on microalgae growth and to detect significant differences between total lengths of emerging hyphae per bead and per Petri dish (containing 5 beads) according to three concentrations of S. dimorphus supernatants in the last experiment testing the impact of microalgal exudates encapsulated with mycorrhizal spores on germinating hyphal elongation.

Finally, to compare results obtained by OD measurement and counting cells of the experiments testing the effect of AM fungal hyphae exudates on microalgae growth, a non-parametric correlation test (Spearman method) was carried out (with the R program).

IV. Results

I. Impact of microalgal exudates on the germination of spores, hyphal branching and elongation of germinating hyphae

I.I Effects of microalgae exudates from 3N-BBM medium

A pre-germination test was conducted with the four strains. Only three (*R. irregularis* MUCL 41833, *R. aggregatum* MUCL 49408 and *R. intraradices* MUCL 49410) showed adequate germination rates and were selected for the experiment. The strain *R. clarus* MUCL 46238 was not selected due to the low germination rate during the pre-test.

A. Germination rate

The germination rate is the percentage of germinated spores at the end of the experiment (i.e. after three weeks).

Table 4: *Germination rate [%] of spores of R. intraradices MUCL 49410, R. irregularis MUCL 41833 and R. aggregatum MUCL 49408 strains with increasing concentrations of S. dimorphus 3N-BBM exudates.*

	Germination rate [%]			
	CTRL	**0.25%**	**2.5%**	**25%**
R.intraracides MUCL 49410	88	89	85	83
R.irregularis MUCL 41833	83	58	92	90
R.aggregatum MUCL 49408	84	69	97	100

For whatever fungal strain, each treatment induced a germination rate above 50%.

B. Germination day

The germination day is the day at which the spore germinated, with day 0 = the day the spore was placed in the Petri dish. A germination day of 4 means the first germination tube was observed at day 4. Data of germination day lacks for the strain *R. irregularis* MUCL 41833.

No significant impact of exudates was noticed for and *R. aggregatum* MUCL 49408 ($p<0.05$) (Table 5). Conversely, exudates induced a significant difference for *R. intraradices* MUCL

49410. At concentration 2.5 %, the germination day was significantly higher as compared to the CTRL and the 0.25% of *R. intraradices* MUCL 49410, but not as compared to the 25% of *R. intraradices* MUCL 49410 and the four treatments of *R.aggregatum MUCL* 49408. No significant difference was noticed in germination day between CTRL, 0.25% and 25% exudates of *R.intraracides* MUCL 49410 and the four treatments of *R.aggregatum MUCL* 49408.

***Table 5** : Germination day of R. intraradices MUCL 49410 and R. aggregatum MUCL 49408 spores with increasing concentrations of S. dimorphus 3N-BBM exudates.*

	Germination Day [Day]			
Conc.[%]	**CTRL**	**0.25%**	**2.5%**	**25%**
R.intraracides MUCL 49410	4.5 ± 6.121^{b}	4.5 ± 6.334^{b}	10.642 ± 5.879^{a}	10.133 ± 7.643^{ab}
R.aggregatum MUCL 49408	7.2 ± 3.3^{ab}	6.8 ± 4.3^{ab}	7.4 ± 6.2^{ab}	8.1 ± 4.7^{ab}

Note: Data are presented as means ± SD (standard deviation). Different lower case letters indicate significant differences between increasing concentrations of S. dimorphus 3N-BBM exudates, as determined by a Kruskal-Wallis test followed by a Tukey's test. ($p<0.05$)

C. Hyphal elongation

In a first trial with *R. intraradices* MUCL 49410, some hyphae attached to the selected spores were particularly long. We hypothesized that this may have impacted the length of germinating hyphae.

Therefore, to ensure that the length of the subtending hypha did not interfere with the results, a non-parametric correlation test between the subtending hypha length and the daily growth of hyphae (after 7 days) was performed. A ρ of -0.27 was obtained. Although this value was low, the correlation was negative. A Kruskal-Wallis test was thus conducted. It showed that the lengths of the subtending hyphae were evenly distributed in the different concentrations (Figure 29). It was therefore decided to not take that into consideration.

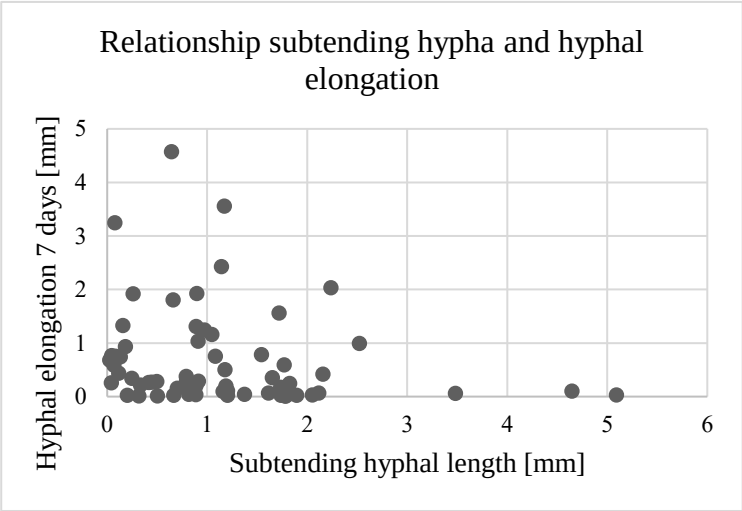


***Figure 27**: Hyphal elongation at 7 days against the length of subtending hypha for R. intraradices MUCL 49410 spores for different concentrations of S. dimorphus 3N-BBM exudates*

Three measures of hyphal elongation were taken in a period of 7 days. Daily growth over the whole 7 days period was derived and the averages of each concentration were reported in Table 6.

***Table 6:** Daily growth [mm/day] during a period of 7 days of hyphae from germinated spores of R. intraradices MUCL 49410, R. irregularis MUCL 41833 and R. aggregatum MUCL 49408 strains with increasing concentrations of S. dimorphus 3N-BBM exudates.*

	Daily Growth [mm/day]			
	CTRL	**0.25%**	**2.5%**	**25%**
R.intraracides MUCL 49410	0.239±0.330^{b}	0.262±0.222^{b}	1.412±1.326^{a}	0.526±0.449^{b}
R.irregularis MUCL 41833	0.318±0.253^{b}	0.263±0.295^{b}	1.502±0.554^{a}	1.554±1.051^{a}
R.aggregatum MUCL 49408	0.258±0.165^{b}	0.284±0.176^{b}	0.462±0.438^{b}	1.365±0.765^{a}

Note: Data are presented as means ± SD. Different lower case letters indicate significant differences between increasing concentrations of S. dimorphus 3N-BBM exudates, as determined by a Kruskal-Wallis test followed by a Tukey test. ($p<0.05$)

Daily growths of *R. intraradices* MUCL 49410 2.5 % exudates, *R. irregularis* MUCL41833 2.5% and 25% exudates and *R.aggregatum MUCL* 49408 25% exudates were significantly higher as compared to the controls and the 0.25% exudates of all three strains, the 25% exudates of *R. intraradices* MUCL 49410 and the 2.5% exudates of *R.aggregatum MUCL* 49408. On a

period of 7 days, the increases were estimated to be about 4 to 5 times as high, by comparison to the controls.

The highest daily growth was induced by the 25% *R.irregularis* MUCL 41833 (1.554 mm/day), followed by the 2.5 % *R.irregularis* MUCL 41833 (1.502 mm/day), the 2.5 % *R.intraracides* MUCL 49410 (1.412 mm/day) and the 25 % *R.aggregatum MUCL* 49408 (1.365 mm/day).

For the strain *R.intraracides* MUCL 49410, daily growths over different time periods (0-2, 2-4, 4-7 and 0-7 days) were calculated and the averages of each concentration were reported in Table 7. For the other two strains, some missing data on days 2 and 4 did not allow to measure daily growths over time.

***Table 7**: Daily growth [mm/day] of hyphae for different periods of 2, 2, 3 and 7 days between day 0 and day 2, day 2 and day 4, day 4 and day 7, day 0 and day 7 respectively after germination of spores of R.intraracides MUCL 49410 with varied concentrations of S. dimorphus 3N-BBM exudates.*

	Daily Growth [mm/day]			
	CTRL	**0.25%**	**2.5%**	**25%**
0-2	0.165 ± 0.243^{b}	0.183 ± 0.244^{b}	1.198 ± 1.634^{a}	0.658 ± 1.141^{ab}
2-4	0.315 ± 0.352^{b}	0.427 ± 0.585^{b}	1.282 ± 1.399^{a}	0.629 ± 1.043^{ab}
4-7	0.240 ± 0.313^{b}	0.200 ± 0.180^{b}	1.630 ± 1.708^{a}	0.383 ± 0.431^{b}
0-7	0.239 ± 0.330^{b}	0.262 ± 0.222^{b}	1.412 ± 1.326^{a}	0.526 ± 0.449^{b}

Note: Data are presented as means ± SD. Different lower case letters indicate significant differences between increasing concentrations of S. dimorphus 3N-BBM exudates, as determined by ANOVA1 followed by a Tukey test. ($p<0.05$)

After two days, there was already a significant difference between hyphal elongation at 2.5% exudates as compared to the CTRL and 0.25% exudates. However, the 2.5% exudates did not induce a significant difference in daily growths compared to the 25% exudates. The CTRL and the 0.25% exudates were not significantly different from each other.
The daily growth between day 2 and day 4 gave the same trends. Daily growth between day 4 and day 7 gave a significant difference between 2.5 % and the three other concentrations. Again, the CTRL and 0.25% exudates on one hand and the 2.5 and the 25% on the other hand, did not induce a significant different daily growth. Between day 4 and day 6, daily growth of treatment 2.5% was significantly different from the three other treatments. The later were not significantly different from each other. This is the same trend than the daily growth taken for the whole 7 days period.
As time progressed, the daily growth of hyphae subjected to treatment 2.5% significantly differentiated from the daily growth of hyphae subjected to the other treatments. First from the CTRL and 0.25% exudates (until day 4) and then from the 25% exudates.

In following Figure 30, which reflects hyphal elongation velocities over time, daily growths of different time periods have been graphically presented. For the four treatments, daily growths at the beginning (from day 0 to day 2), the middle (from day 2 to day 4), the end (from day 4 to day 7) or for the whole 7 days are not significantly different from each other. From day 0 to day 2, there is in general less diversity in daily hyphal elongation between the replicates of the same treatment than from day 2 to day 4 and day 4 to day 7.

Boxplots of daily hyphal elongation over different time periods for different concentrations of supernatants

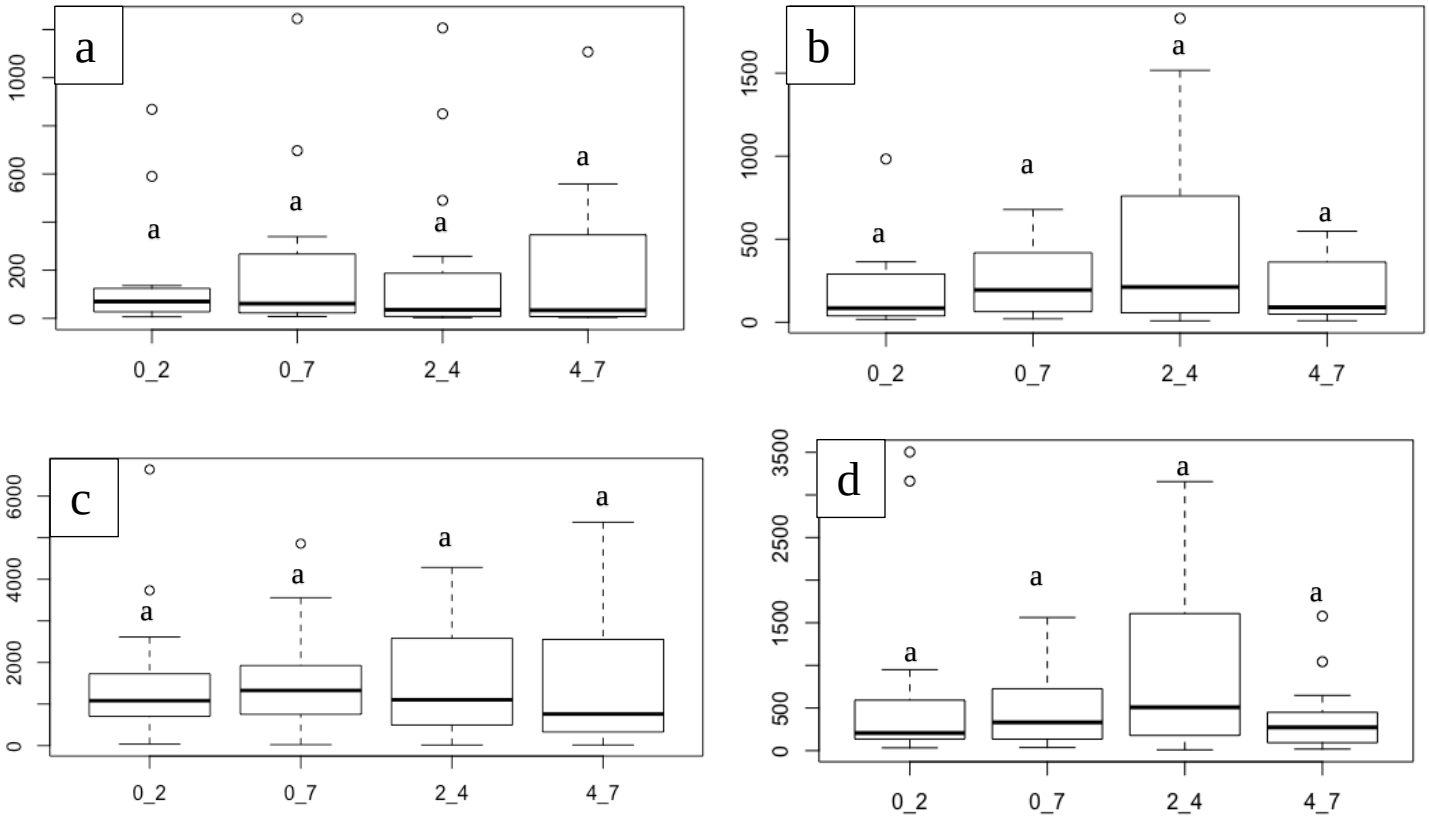


***Figure 28**: Boxplots of daily hyphal elongation over different time periods for different concentrations of supernatants. On the x-axis the time period [-]: from day 0 to day 2, from day 2 to day 4, from day 4 to day 7 and from day 0 to day 7 and on the y-axis the daily hyphal elongations [10^{-3} mm/day]. The graph a represents the concentration 0, the graph b the concentration 0.25, the graph c the concentration 2.5 and the graph d the concentration 25. Different letters indicate significant differences between daily hyphal elongation over different time periods, as determined by an ANOVA1 followed by a Tukey test. ($p<0.05$)*

C. Hyphal branching

Three measures of hyphal branching were taken during a period of 7 days for the three strains (*R. irregularis* MUCL 41833, *R. aggregatum* MUCL 49408 and *R. intraradices* MUCL 49410). Daily ramification over the whole 7 days period was derived and the averages of each concentration were reported in Table 10.

For *R. intraracides* MUCL 49410 no significant difference between treatments was noticed in the daily ramification of the growing hyphae. Daily ramification of *R.irregularis* MUCL 41833 and *R.aggregatum MUCL* 49408 25% exudates treatment was significantly higher than all the other treatments, except from the *R.irregularis* MUCL 41833 2.5% exudates treatment. This latter treatment was significantly higher than the three CTRL and 0.25% treatments and *R.aggregatum MUCL* 49408 2.5% treatment. There is no significant difference perceptible between the controls and the 0.25% treatments of the three strains.

Table 8: *Daily ramification [Ram/day] for a period of 7 days of hyphae from germinated spores of R. intraradices MUCL 49410, R. irregularis MUCL 41833 and R. aggregatum MUCL 49408 strains with varied concentrations of S. dimorphus 3N-BBM exudates.*

	Daily Ramification [Ram/day]			
	CTRL	**0.25%**	**2.5%**	**25%**
R.intraracides MUCL 49410	0.443 ± 0.183^{c}	0.567 ± 0.235^{c}	0.619 ± 0.395^{bc}	0.590 ± 0.365^{bc}
R.irregularis MUCL 41833	0.333 ± 0.144^{c}	0.367 ± 0.108^{c}	1.0816 ± 0.681^{ab}	1.333 ± 0.914^{a}
R.aggregatum MUCL 49408	0.153 ± 0.0382^{c}	0.162 ± 0.0911^{c}	0.299 ± 0.0811^{c}	1.296 ± 0.539^{a}

Note: Data are presented as means ± SD. Different lower case letters indicate significant differences between increasing concentrations of S. dimorphus 3N-BBM exudates, as determined by a Kruskal-Wallis test followed by a Tukey test. ($p<0,05$). Ram= Ramifications

I.II Effects of microalgae exudates from MSR(+ and -) medium

S. dimorphus and *C. vulgaris* were incubated in the dark and under light conditions to compare the effects of exudates on spore germination, hyphal elongation and branching. Unfortunately, the exudates of *S. dimorphus* cultivated in the dark were lost. Data are thus only available for *C. vulgaris* in the light and in the dark and *S. dimorphus* in the dark.

Results were displayed in two tables in order to compare the exudates of *C. vulgaris* cultivated in the dark and in the light (Table 9) on one side and the exudates of *C. vulgaris* and *S. dimorphus* cultivated in the dark on the other side (Table 10). The results of the control and the exudates of *C. vulgaris* cultivated in the dark appear in both tables. The tables present the germination rate 30 days after the experiment was launched, the averages of spore (*R. irregularis* MUCL 41833) germination days and the averages of daily growth and daily ramification obtained after 7 days of spore germination are for the different treatments.

***Table 9**: Germination rate (%) of spores of R. irregularis MUCL 41833 over 30 days, germination day (day), daily growth (mm/day) and daily ramification (Ram./day) for a period of 7 days of hyphae from the germinated spores with increasing concentrations of MSR (+ and -) exudates of C. vulgaris cultivated in light (MSR-) and dark (MSR+) conditions.*

			***R. irregularis* MUCL 41833**			
			Germination Rate [%]	**Germination Day [Day]**	**Daily Growth [mm/day]**	**Daily Ramification [Ram./day]**
C. vulgaris		CTRL	75	10.933±5.812[a]	0.0528±0.120[c]	0.167±0.0610[c]
	light	0.25%	33	10.667±9.852[a]	0.0144±0.005[c]	0.146±0.0510[c]
		2.5%	5.6	3[b]	0.0144[c]	0.125[c]
		25%	84	4.750±9.852[a]	0.847±0.631[a]	0.625±0.309[a]
	dark	0.25%	35	10.00±7.885[a]	0.071±0.0607[bc]	0.188±0.0685[bc]
		2.5%	0	0	0	0
		25%	67	7.00±6.769[a]	0.507±0.295[ab]	0.385±0.164[b]

Note: Data are presented as means ± SD. Different lower case letters indicate significant differences between increasing concentrations of exudates, as determined by Kruskal-Wallis test followed by a Tukey test. ($p<0.05$) Ram.= Ramifications

The treatments 0.25% and 2.5% of *C. vulgaris* exudates cultivated in the light and in the dark induced germination rates below 50% (33%, 5.6%, 35%, 0% respectively).

Exudates of *C. vulgaris* grown in MSR- medium in the light induced a significantly earlier germination on the spores added at a concentration of 2.5%, as compared to the control, the 0.25% and the 25% of both *C. vulgaris* grown in MSR+ in the dark and in MSR- medium in the light. The five latter treatments were not significantly different from each other.

Added at the concentration of 25%, the exudates of *C. vulgaris* cultivated in the light and in the dark induce a significant increased daily hyphal growth in comparison to the control, *C. vulgaris* cultivated in the light 0.25% and 2.5% treatment. The 25% exudates of *C. vulgaris* cultivated in the light end in the dark are not significantly different from each other and the daily growth they induced is estimated to be respectively about 16 and 9 times as high, as compared to the control. The control, the 0.25% and the 2.5% treatments of *C. vulgaris* cultivated in the light on one side and the 0.25% and the 25% treatments of *C. vulgaris* cultivated in the dark on the other side did not induce a different daily growth.

Concerning the daily hyphal ramification, the treatments induced the same significant differences than for the daily hyphal growth, except that the exudates from *C. vulgaris* cultivated in the light induced a significantly higher ramification than the exudates from *C. vulgaris* cultivated in the dark at the 25% concentration (about 1.6 times as high).

***Table 10**: Germination rate (%) of spores of R. irregularis MUCL 41833 over 30 days, germination day (day), daily growth (mm/day) and daily ramification (Ram./day) for a period of 7 days of hyphae from the germinated spores with increasing concentrations of MSR (+ and -) exudates of C. vulgaris and S. dimorphus in dark (MSR+) conditions.*

		***R. irregularis* MUCL 41833**			
		Germination Rate [%]	**Germination Day [Day]**	**Daily Growth [mm/day]**	**Daily Ramification [Ram./day]**
	CTRL	75	10.933±5.812[a]	0.0528±0.120[b]	0.167±0.0610[b]
C. vulgaris	0.25%	35	10.00±7.885[a]	0.071±0.0607[b]	0.188±0.0685[b]
	2.5%	0	0	0	0
	25%	67	7.00±6.769[a]	0.507±0.295[a]	0.385±0.164[a]
S. dimorphus	0.25%	78	10.286±6.305[a]	0.0401±0.0356[b]	0.152±0.0724[b]
	2.5%	22	9.250±4.088[a]	0.0121±0.00538[b]	0.156±0.0625[b]
	25%	90	6.118±0.111[a]	0.600±0.1626[a]	0.375±1.799[a]

Note: Data are presented as means ± SD. Different lower case letters indicate significant differences between increasing concentrations of exudates, as determined by Kruskal-Wallis test followed by a Tukey test. ($p<0.05$) Ram.= Ramifications

As regards the microalgal exudates of *S. dimorphus* grown in MSR+ medium in the dark, only treatment 2.5% induced a germination rate below 50 % (22%).

There was no significant difference in germination day between the control and the 6 *C. vulgaris* and *S. dimorphus* treatments cultivated in the dark.

Treatments 25% of *C. vulgaris* and *S. dimorphus* cultivated in the dark induced a significantly higher daily hyphal growth and branching as compared to the control, the 0.25% treatments of *C. vulgaris* and *S. dimorphus* cultivated in the dark and the 2.5% treatment of *S. dimorphus* cultivated in the dark. The latter treatments were not significantly different from each other.

New experiences were launched with the 5 treatments that had a germination rate below 50 % and a control, in a view to clarify whether the exudates had an inhibitory effect on germination or whether another factor had played a role. The table displaying those results can be found in appendix 4. Unfortunately, the controls of the second experience had zero percent germination rate. Germination rates of the other treatments were also low and under 50%, except the germination rate of the 2.5% treatment of *S. dimorphus* cultivated in the dark, which was 57.9%. As the germination rate of the control was 0, no statistics could be done and the results are therefore not useable.

II. Effects of microalgal exudates encapsulated with mycorrhizal spores on germination of spores and germinating hyphal elongation

This experiment was set up to test the impact of microalgal exudates encapsulated with mycorrhizal spores on germination of spores and germinating hyphal elongation. A good germination was observed for all the beads (containing 0%, 10% or 100% microalgal MSR-exudates).

After four days, total lengths of the emerging hyphae per bead were calculated (Table 21). The total hyphal length per bead containing 10% of *C. vulgaris* exudates was significantly higher (about more than twice as high) in comparison to the total hyphal length per bead containing 0% and 100% exudates. The latter treatments were not significantly different from each other.

***Table 11**: Total length of emerging hyphae per bead according to three concentrations of supernatant from S. dimorphus after 4 days.*

	Concentration microalgal exudates [%]		
	0%	**10%**	**100%**
Total hyphal length per bead [µm]	1782 ± 2234[a]	4277 ± 3062[b]	1991 ± 2672[a]

Note: Data are represented as means ± SD with n = 50 per treatment. Different letters indicate significant differences between the 0%, 10% and 100% treatments, as determined by an ANOVA1 followed by a Tukey test. (p<0,05)

Lengths of the total hyphae emerging inside Petri dishes including 5 beads were also measured after 4 days (Figure 31). From this point of view, a concentration of 10% microalgal exudates induced also a significantly higher hyphal elongation per Petri dish, as compared to the 0% and the 100% exudates. The latter treatments were not significantly different from each other.

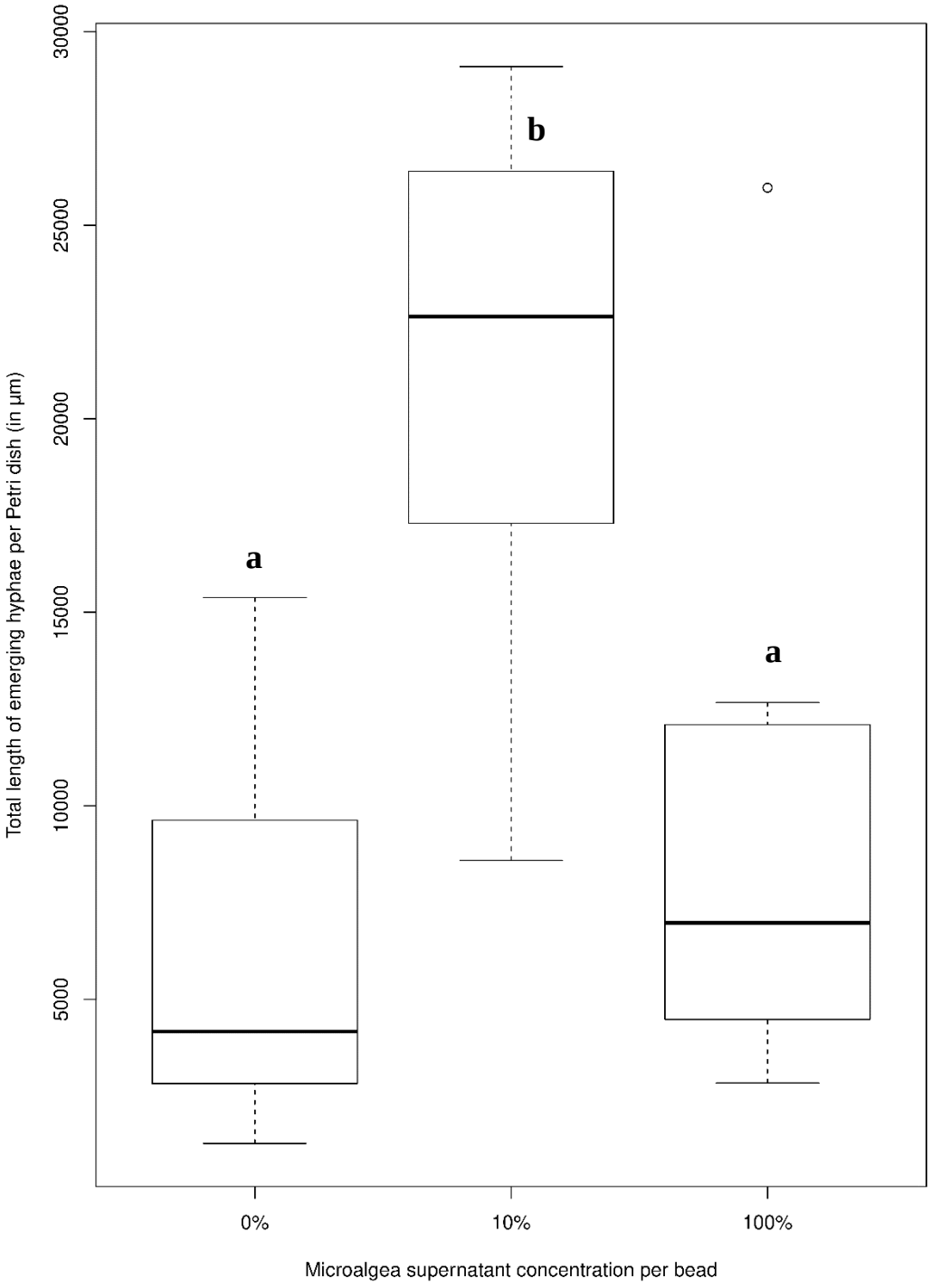


***Figure 29**: Length (µm) of the total hyphae of the strain R. irregularis MUCL 41833 emerging inside Petri dishes (including 5 beads), according to beads including three supernatant concentrations (0, 10 and 100%) of S. dimorphus. Different letters indicate significant differences between the 0%, 10% and 100% treatments, as determined by an ANOVA1 followed by a Tukey test. (p<0,05) Each boxplot includes n = 10 Petri dishes.*

III. Effects of AM fungal exudates on microalgae growth

III.I Preliminary assays

A. Determination of adequate dilution to stimulate microalgae growth

Several dilutions (1/100, 1/1000, 1/10 000, 1/100 000) were tested on *S. dimorphus* growth in Petri dishes containing 3N-BBM medium (agar 6g/L). In a first assay, dilutions 1/10 000 and 1/100 000 appeared to be the most adequate. The dilutions permitted to have a few distinct colonies and to have a good visualisation of their development. But the results could not always be reproduced and it appeared to be too diluted sometimes, even though cells were collected at the same time after latest change of medium. Therefore there was decided to apply the microalgae at a dilution 1/1000 on the Petri dishes of the final experiments.

B. Effects of streptomycin on *S. dimorphus* growth

Zero, 0.12, 0.24 and 2.4 of streptomycin concentrations were tested on the growth of *S. dimorphus* (table 12) in an attend to inhibit photosynthesis and to detect how microalgae develop in heterotrophic conditions (color, growth ,…).

Table 12 *:Effects of different streptomycin concentrations on S. dimorphus growth in gelled MSR- medium.*

Conc [mg/l]	**0**	**0.12**	**0.24**	**2.4**
Growth	Normal	Normal	Slightly reduced	reduced

Development of *S. dimorphus* was normal when the gelled MSR- medium contained 0.12mg/L streptomycin. At a concentration of 0.24 mg/L, the growth was slightly reduced while at 2.4 mg/L, growth was markedly reduced. It was thus decided to make further experiments with streptomycin 2.4 mg/L.

B. Growth of *S. dimorphus* in heterotrophic and mixotrophic cultures

With these experiments, we wanted to know if *S. dimorphus* could be cultivated heterotrophically and mixotrophically, with sucrose 10 g/L or glucose 1g/L and 10g/L in both liquid and gelled medium.

- Liquid cultures

After 3 weeks, the number of microalgae cells was evaluated with a Fuchs-Rosenthal cell. The best development of *S. dimorphus* was observed in the MSR+(glucose 1g/L) culture, followed by the MSR+(sucrose 10g/L) culture, the MSR+(glucose 10g/L) culture and finally the MSR- in the dark. The concentration of microalga, between day 1 and day 21, increased in all cultures except the MSR – culture.

- First Petri dishes cultures

After 10 days, *S. dimorphus* growth on the Petri dishes was observed. Following table 13 was made to support the observations. This table is subjective, no results were calculated. It approximates the reality and gives the observer's impression. Averages of the replicates of the development of *S. dimorphus* are given in the form of +'s and zeros.

***Table 13**: Development of S. dimorphus on MSR -, MSR+S (10 g/L sucrose), MSR+G1 (1g/L glucose) or MSR+G10 (10g/L glucose) medium with or without streptomycin (2.4 mg/L) in the light or in the dark.*

	Growth			
	Light		**Dark**	
	Streptomycin	**--**	**Streptomycin**	**--**
MSR -	++	++ to +++	0 to +	0 to +
MSR + S [10g/L sucrose]	+	+++	0 to +	0 to +
MSR + G10 [10g/L glucose]	0 to +	0 to +	+	0 to +
MSR + G1 [1g/L glucose]	+++ to ++++	++++	0 to +	+

0 No Development
+ Low Development
++ Medium development
+++ Good development
++++ Really good development

All the Petri dishes that were in the dark showed no to very small green spots. Unfortunately, bigger orange and white colonies (contaminations) were also observed (Figure 32). For the Petri dishes that had incubated in the light, the best growth was observed for MSR + G1, followed by MSR+S and MSR-. MSR+G1 and MSR+S had bigger green spots, while the spots of MSR - where often more numerous and smaller. A bad development for all the MSR+G10 Petri dishes was observed. There was a small perceptible difference between the Petri dishes with and without streptomycin, but colonies still developed on Petri dishes with streptomycin. The duplicates were not exact copies, although the same dose was applied and the same conditions were fulfilled.

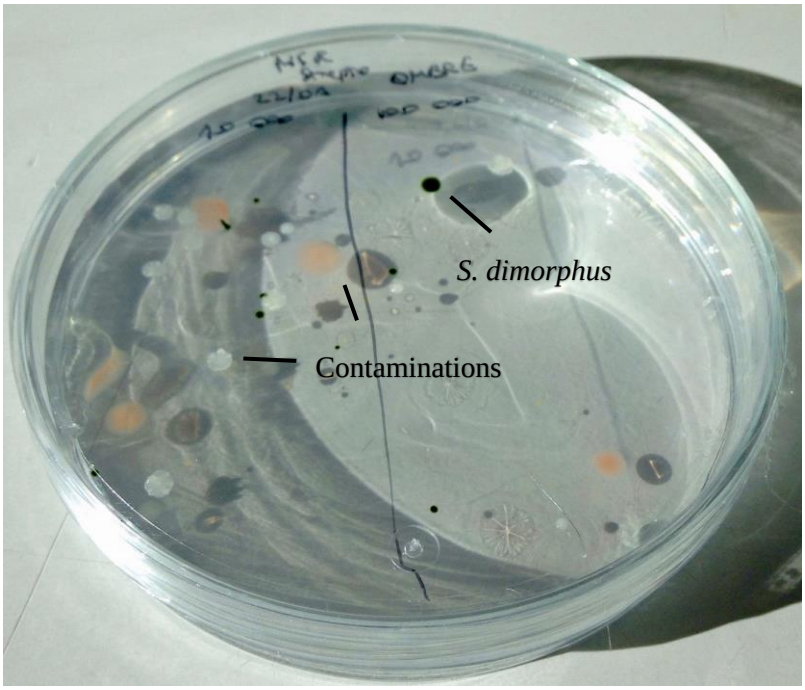


***Figure 30**: Petri dish of 1/10 000 and 1/100 000 diluted 10 µL spots of S. dimorphus on MSR- medium incubated in the dark after 10 days.*

Although the Petri dishes contained contaminations, the results were interesting. As a first, it allowed to see that *S. dimorphus* did not grow well with glucose 10 g/L. Secondly, streptomycin didn't seem to inhibit growth of *S. dimorphus* enough. As the morphology and color of *S. dimorphus* in heterotrophic conditions was not known, the doubt that the white spots could be *S. dimorphus* colonies was there. A new experiment was therefore launched, without the glucose 10 g/L and the streptomycin, after purification of the microalgal culture.

- Second Petri dishes cultures

After 2 weeks, *S. dimorphus* growth on the Petri dishes was observed. Similarly as the first Petri dishes culture, a table (Table 13) was made to support the observations.
After 2 weeks, growth of *S. dimorphus* on the distinct Petri dishes was observed. The ones that had incubated in the light, plus MSR + S and MSR+ G1 in the dark, looked very green. MSR+ G1 in the light and in the dark looked slightly greener than MSR+S in the light and in the dark and they all looked greener than MSR- in the light. No real distinction could be made between MSR+ S in the light and in the dark on one side and MSR+ G1 in the light and in the dark on the other side. On the MSR- Petri dishes from the dark, nothing was perceptible to the naked eye. This time, no other colors were distinguishable, so the white colonies observed in the dark Petri dishes in the first Petri dishes culture were contaminations and not *S. dimorphus*. This time there was no doubt: *S. dimorphus* can be cultivated in heterotrophic and mixotrophic conditions with glucose 1 g/L and sucrose 10 g/L and it does not grow well if no sugar is added in the dark. In general, glucose 1g/L provided the best microalgae growth, followed by sucrose 10 g/L in the dark especially and also in the light (Figure 30).

***Table 14**: Development of S. dimorphus on MSR -, MSR+S (10 g/l sucrose) or MSR+G1 (1g/l glucose) in the light or in the dark.*

	Growth	
	Light	**Dark**
MSR -	+++	0
MSR + S [10g/l sucrose]	+++ to ++++	+++ to ++++
MSR + G1 [1g/l glucose]	++++	++++

0 No Development
+ Low Development
++ Medium development
+++ Good development
++++ Really good development

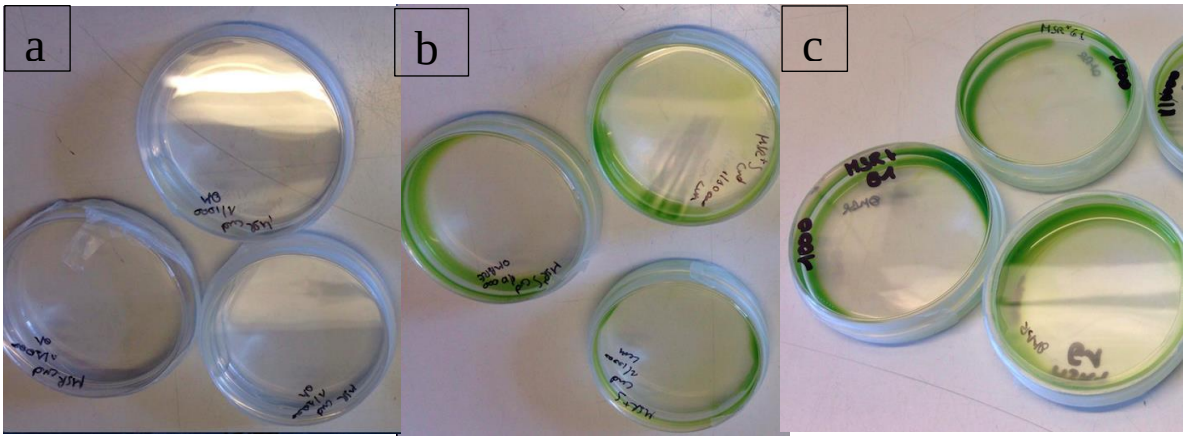


***Figure 31** : Petri dishes of 1/1000 diluted 100 µl spots of S. dimorphus on MSR-* (a), *MSR+S* (b) *and MSR+G1* (c) *incubated in the dark after 2 weeks*

E. Interaction between AM fungal hyphae and microalgae in heterotrophic conditions on solid medium

Now that it had been determined that *S. dimorphus* could grow under heterotrophic conditions in a liquid and gelified medium, that it was known that it could feed on glucose, a by hyphae exuded sugar, and that a clear difference was observable between cultures with and without sugar added in the dark, the next step was to see if hyphae could be its sugar supply.

The experiment was conducted with *R. irregularis* MUCL 41833 and *S. dimorphus*. After one week of growth of *S. dimorphus* in the AM fungal mycelium on the MSR- medium in the HC, colonies could be observed for the two Petri dishes that had incubated under light conditions.

Some small spots could also be observed on the MSR- medium with and without hyphae that had incubated in the dark.

After two weeks, colonies could be observed with the naked eye for the Petri dishes that had incubated in the light. Under the microscope, big colonies were observed. The best development was observed in the HC containing no hyphae (Figure 35). As regards the Petri dishes that had incubated in the dark, no colonies were observed with the naked eye on, with and without hyphae in the hyphal compartment (Figure 35). Under the microscope, the Petri dishes containing hyphae had developed into colonies of a few cells (Figure 34). For the Petri dishes without hyphae, the microalgal cells had developed into bigger cells and some colonies of a few cells were observed. The development was very poor.

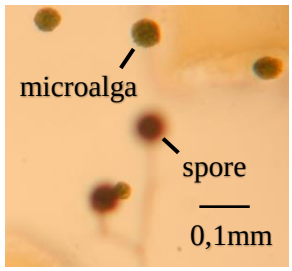


***Figure 32**: Development of S. dimorphus after two weeks in the dark on MSR- medium with hyphae of R. irregularis MUCL 41833*

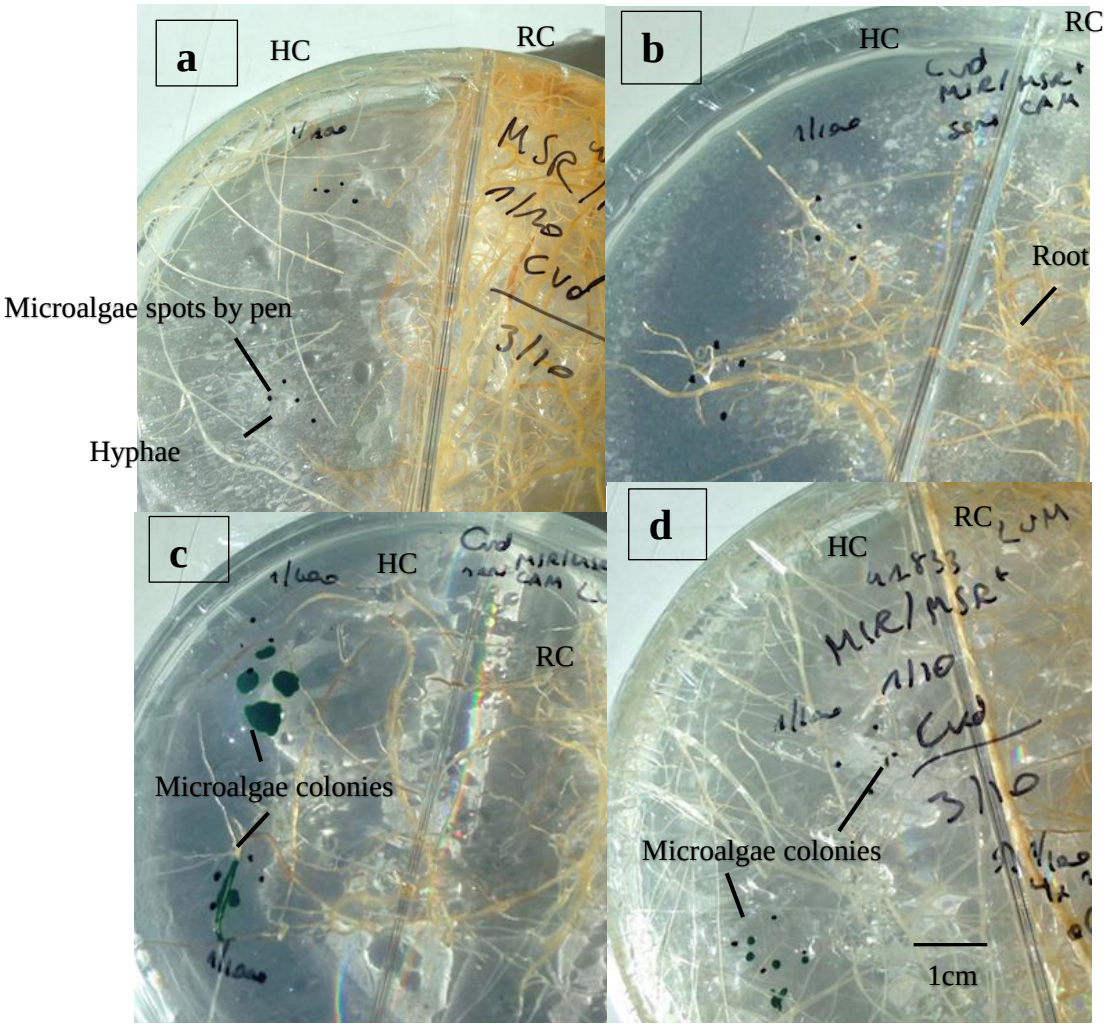


***Figure 33:** S. dimorphus spots in the HC (MSR- medium) containing hyphae of the AM fungus R. irregularis MUCL 41833.The Petri dishes were incubated in the dark (a) or in the light (d). HC without hyphae were also considered incubated in the dark (b) or in the light (c).*

Furthermore, the microalgae seemed to tend to follow the hyphae and the roots (Figure 36).

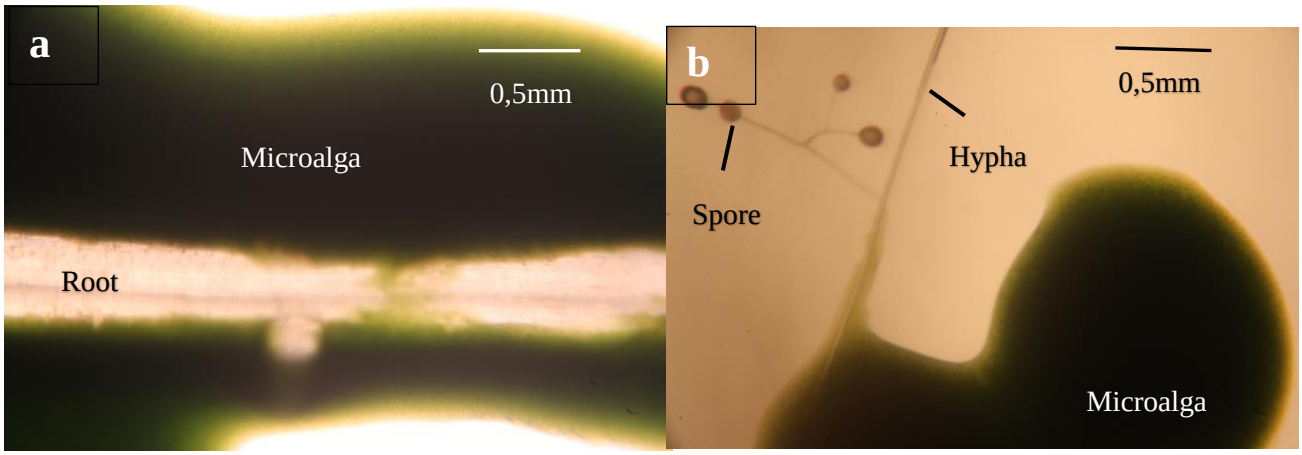


***Figure 34**: S. dimorphus cells following a root (a) and a R. clarus MUCL 46238 hypha (b) in MSR-medium after two weeks of incubation in the light*

The observation of microalgae following hyphae in the HC in the light four weeks later (i.e. 6 weeks after application of *S. dimorphus*) allowed to see that *S. dimorphus* had not developed further and no further migration along the hyphae was observed (Figure 37). Moreover, on the following figure 37 a contamination appeared.

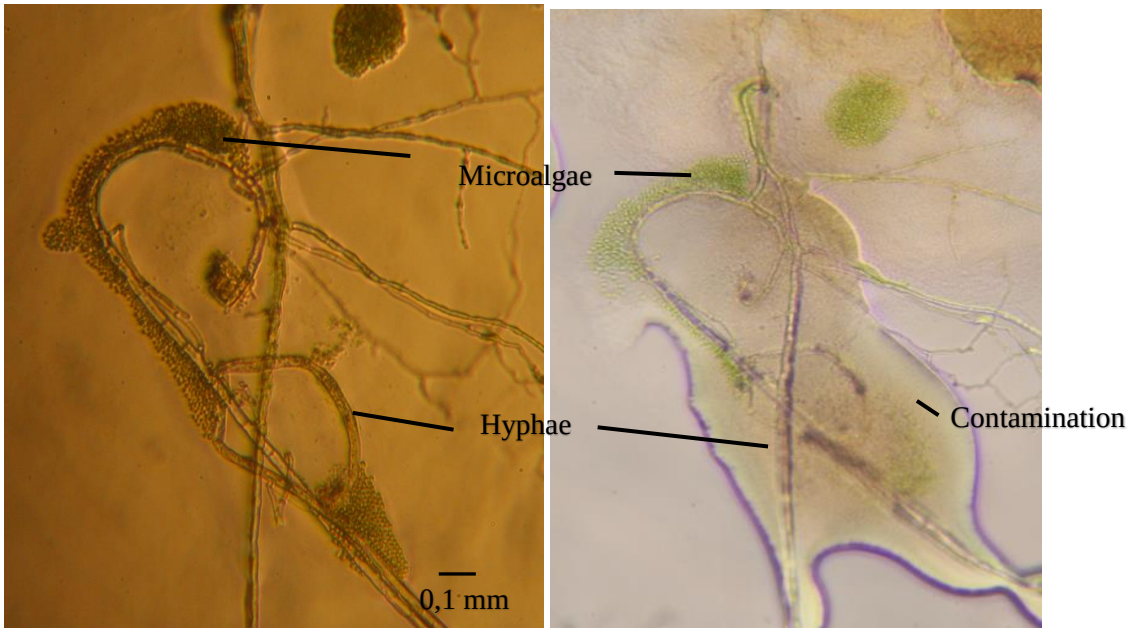


***Figure 35**: Microscopic picture of S. dimorphus along R. irregularis MUCL 41833 hyphae on MSR- medium after 2 weeks and 6 weeks*

This pretest showed that *S. dimorphus* grew much better in the presence of light than in the absence of light, in the presence of AM fungal hyphae and roots or not. In the presence of light, *S. dimorphus* had a better grow in the absence of AM fungal hyphae and roots. In the dark, *S. dimorphus* growth was very small in comparison with its development in the light, but it seemed that *S. dimorphus* grew better in the presence of AM fungal hyphae and roots. This assay had

promising results, as *S. dimorphus* seemed to be able to grow better in the dark in the presence of AM fungal hyphae than in the absence of AM fungal hyphae, but the roots in the HC and the limited number of replicates did not allow to draw any conclusion.

III.II Final experiments

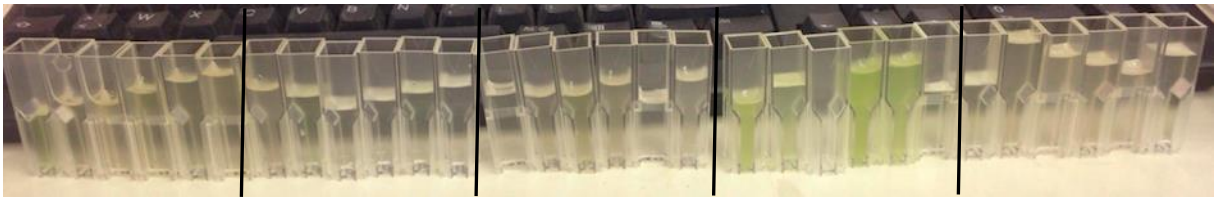
The main objective of these experiments is to observe if microalgae can prosper in a whole dark environment with no source of sugars, except the one that can potentially be given by mycorrhizae. Three experiments were set-up. In the first one, HC is composed of liquid medium. In the latter two HC is composed of solid medium.

A. Experiment with liquid hyphal compartment

Microscopic observations

Before analyzing the Petri dishes under the microscope, a few things were noted to the naked eye. The first one was that the remaining volumes of the liquid compartments were very different. At the end of the experiment, the smallest and largest remaining volumes were 5 and 8.9 ml, for an initial volume of 10 ml. However, within the same treatment volumes were quite heterogeneous. The means and standard deviation of the volumes for each treatment are represented in Table 11 below. Different treatments induced no significant difference in the remaining final volumes.

Secondly, some Petri dishes looked much greener than others. Intensities of green of the liquid compartments were not uniform within the same group. For treatment C (control with no roots no AM fungus in the RC), microalgal culture of 3 out of the 6 Petri dishes look very green compared to the microalgal culture in the latter three. The Petri dishes of treatment S (control with roots and AM fungus in the RC) are the least green overall (Figure 35).



41833 S **41833 E** **49410 S** **C** **49410 E**

***Figure 36**: Samples of microalgal compartments volume after 13 days of incubation in the dark in the liquid MSR compartment for the C (microalgae in the HC/ no roots no mycorrhizae in HC and RC) , S (microalgae in the HC/ roots and mycorrhizae in the RC) and E (microalgae and mycorrhizae in the HC/ roots and mycorrhizae in the RC) treatments of R. irregularis MUCL 41833 and R. intraradices MUCL 49410 strains.*

In addition, it was observed that microalgae were not evenly distributed in the whole liquid volume. The content was homogenized using a tip before sampling for the counting tests and the spectrophotometry measurements.

Under the microscope, we observed that hyphae accumulated in the liquid compartment close to the partition wall separating the RC from the HC. The volume occupied by hyphae/spores was thus very small and only a small percentage of microalgae were in contact with the AMF fungal hyphae.
Interestingly, these hyphal clusters were in most cases strongly surrounded by microalgae. In and around these clumps of hyphae and spores, a generally higher concentration of algal cells was noticed as compared to the rest of the compartment (Figure 39).

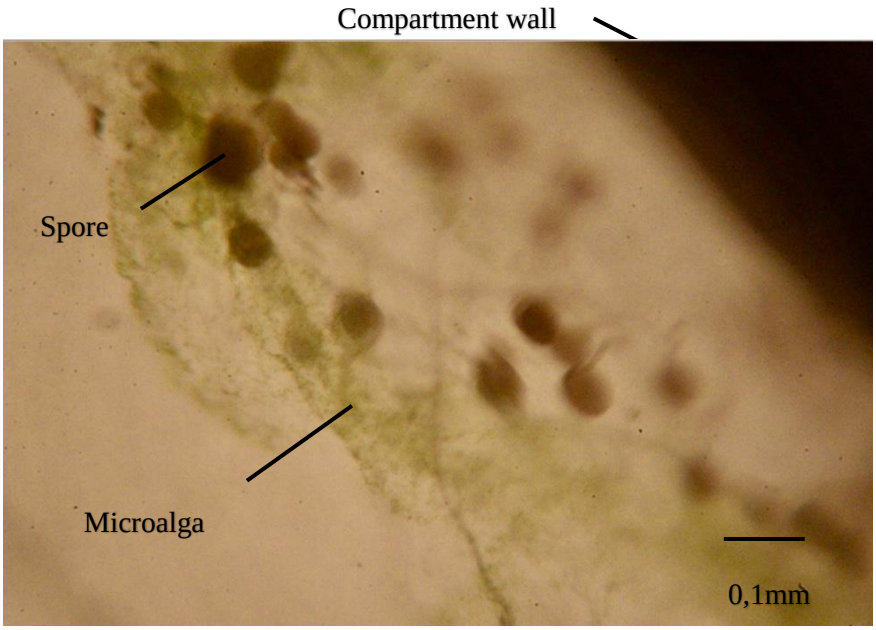


***Figure 37**: Abundant development of C. vulgaris cells along a cluster of R. irregularis MUCL 41833 spores and hyphae on the compartment wall of an E (treatment with microalgae and mycorrhizae in the HC and roots and mycorrhizae in the RC) Petri plate.*

A second observation was the deposit of microalgae on the bottom of the liquid medium (Figure 40). This was not observed throughout the compartment and was even absent in some Petri dishes. The same applies to the accumulation of microalgae along the walls, especially along the one separating the the RC from the HC. In some compartments, a deposit could be observed on the compartment wall, while in others not at all. It was the case in most Petri dishes containing AM fungal hyphae.

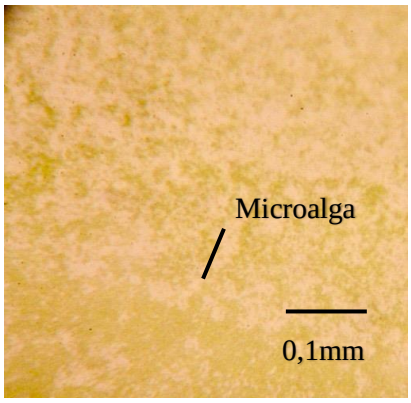


***Figure 38**:Deposit of C. vulgaris on the bottom of a Petri dish*

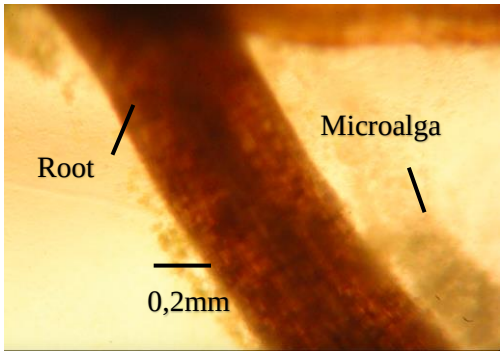


***Figure 39**:C. vulgaris from the liquid MSR compartment of a S Petri dish that ended up in the root compartment by mistake and developed along a DC root after 13 days of incubation in the dark*

OD measurements

Samples were collected at the end of the experiment, on day 13. Absorbance of the samples was measured at 550 and 680 nm wavelengths. Results obtained with wavelengths 550 nm and 680 nm were similar (Table 15). A non-parametric correlation test between the two sets of results provided a ρ of 0.97, which means they are strongly correlated.

***Table 15**: DO_{550} and DO_{680} realized with a DU 800 Spectrophotometer of the hyphal compartment and final volume after 13 days of incubation in the dark of the C (microalgae in the HC/ no roots no mycorrhizae in HC and RC) , S (microalgae in the HC/ roots and mycorrhizae in the RC) and E (microalgae and mycorrhizae in the HC/ roots and mycorrhizae in the RC) treatments (with C. vulgaris, R. irregularis MUCL 41833 and R. intraradices MUCL 49410 strains).*

		Absorbance		
		DO_{550}	**DO_{680}**	**Volume [ml]**
R. irregularis MUCL 41833	C	6347.48±7077.16[a]	6754.80±7543.08[a]	6.62±1[a]
	S	0.51±0.15[b]	0.41±0.13[b]	7.7±1.055[a]
	E	0.23±0.19[b]	0.19±0.14[b]	6.33±1.47[a]
R. intraradices MUCL 49410	C	6347.48±7077.16[a]	6754.80±7543.08[a]	6.62±1[a]
	S	0.39±0.14[b]	0.30±0.11[b]	7.25±1.88[a]
	E	0.32±0.16[b]	0.25±0.12[b]	6.03±1.57[a]

Note: Data are represented as means ± SD. Results are those obtained as such, not adjusted to the volume. Different letters indicate significant differences between C, S and E Petri dishes of the same strain and the same wavelength, as determined by an ANOVA1 followed by a Tukey test. ($p<0,05$)

***Table 16**: Algal biomass obtained from the DO_{550} results and adjusted to the remaining volumes, after 13 days of incubation in the dark of the C (microalgae in the HC/ no roots no mycorrhizae in HC and RC) , S (microalgae in the HC/ roots and mycorrhizae in the RC) and E (microalgae and mycorrhizae in the HC/ roots and mycorrhizae in the RC) treatments (with C. vulgaris, R. irregularis MUCL 41833 and R. intraradices MUCL 49410 strains).*

		Algal biomass X [g/l]
R. irregularis MUCL 41833	C	1385,972±1598,823[a]
	S	0,105±0,0472[b]
	E	0,0577±0,0440[b]
R. intraradices MUCL 49410	C	1385,97±1598,82[a]
	S	0,0944±0,0609[b]
	E	0,155±0,0459[b]

Note: Data are represented as means ± SD. Different letters indicate significant differences between C,S and E Petri dishes of the same strain and the same wavelength, as determined by an ANOVA1 followed by a Tukey test. ($p<0,05$)

Absorbance was converted into algal biomass and the concentrations were adjusted to the remaining volume (Table 16).

The C treatment induced a significant higher absorbance (the results obtained suggest it to be about more than 10 000 times as high) as compared to the S and E treatments of both strains *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410.

Counting tests

C. vulgaris concentrations obtained with the counting tests were adjusted to the remaining volumes (Table 17).

***Table 17**: Concentrations of C. vulgaris obtained by mean of a Fuchs-Rosenthal counting cell after 7,9,11 and 13 days of incubation in the dark in the liquid MSR compartment for the C (microalgae in the HC/ no roots no mycorrhizae in HC and RC) , S (microalgae in the HC/ roots and mycorrhizae in the RC) and E (microalgae and mycorrhizae in the HC/ roots and mycorrhizae in the RC) Petri dishes of R. irregularis MUCL 41833 and R. intraradices MUCL 49410 strains.*

		Conc.[10^9 cells/l]			
Sampling day		**7**	**9**	**11**	**13**
R. irregularis MUCL 41833	C	0.51 ± 0.18^{c}	1.50 ± 0.41^{bc}	3.10 ± 1.14^{b}	7.47 ± 3.15^{a}
	S	0.39 ± 0.18^{c}	0.59 ± 0.33^{c}	0.69 ± 0.47^{c}	1.27 ± 1.25^{bc}
	E	0.4 ± 0.16^{c}	0.79 ± 0.32^{c}	1.051 ± 0.34^{bc}	1.89 ± 1.36^{bc}
R. intraradices MUCL 49410	C	0.51 ± 0.18^{c}	1.50 ± 0.41^{bc}	3.10 ± 1.14^{b}	7.47 ± 3.15^{a}
	S	0.19 ± 0.064^{c}	0.56 ± 0.37^{c}	0.81 ± 0.43^{bc}	2.13 ± 1.23^{bc}
	E	0.35 ± 0.085^{c}	0.71 ± 0.26^{c}	0.64 ± 0.37^{c}	2.5 ± 2.41^{bc}

Note: Data are represented as means ± SD. Different letters indicate significant differences between C, S and E Petri dishes and sampling days 7,9,11 and 13 of the same strain, as determined by a Kruskal-Wallis test followed by a Tukey test. ($p<0,05$)

For both *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410, statistics indicated the same significant differences on sampling day 7, 9 and 13. On day 7 and day 9, there was no significantly different microalgal concentration in the three treatments. The treatments also didn't induce different microalgal concentration between those two sampling days. On sampling day 13, microalgal concentration of the C treatment was significantly different from the two other treatments of that sampling day and the three treatments of sampling days 7, 9 and 11. The S and E treatments were not significantly different from all the treatments of sampling days 7, 9 and 11. For *R. irregularis* MUCL 41833, on sampling day 11 the C treatment was significantly different from the C treatment on sampling day 13, the S treatment on sampling day 11, the S and E treatments on sampling day 9 and the three treatments on sampling day 7. For *R. intraradices* MUCL 49410, on sampling day 11 the C treatment was significantly different from the C treatment on sampling day 13, the E treatment on sampling day 11, the S and E treatments on sampling day 9 and the three treatments on sampling day 7.

The S and E treatments did not induce a significant different microalgal concentration over time (from day 7 to day 13). In contrast, microalgal concentration of treatment C on day 13 was significantly higher than the microalgal concentration of treatment C on the other 3 days and on day 11 it was significantly higher than on day 7.

Sometimes, microalgae form clusters on the Fuchs-Rosenthal cell. Not only does this make counting not easy, but it proves that microalgae, even on Foch-Rosenthal cells, are not evenly distributed. This reflects the importance of counting 18 squares per sample (Figure 41).

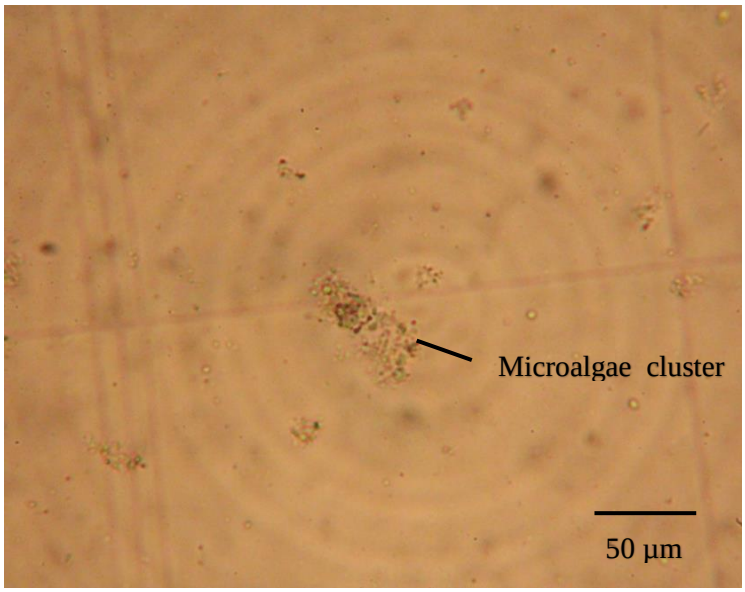


***Figure 40**: C. vulgaris cells forming a cluster on a Fuchs-Rosenthal cell*

Comparison of the methods

Whatever the method, the results are going in the same direction. What is common to the methods is that the Petri dishes of the C group encountered a better development than the other Petri dishes. A second commonality is that the proliferation of microalgae is heterogeneous within the same group and even within the same compartment.

A non-parametric correlation test (Spearman method) between the mass concentrations obtained by OD_{550} and the cellular concentrations obtained by counting tests at the end of the experiment was carried out (Figure 42). A ρ of 0.245 was obtained. When one takes a look at the plots (Figure 42) with the counting tests results in x-axis and the OD results in y-axis, we observed for the first plot, that the 3 tremendous high results pull all the other results on the same line in the y-axis. The second plot, which is the same plot with the 3 high OD results left aside, reports on the non-linear link between the rest of the OD_{550} and the counting results. The same correlation test was carried out on the same data, minus the results obtained for those 3 replicas. A ρ of 0.0269 was obtained.

Mass concentrations obtained by OD against cell concentrations obtained by counting tests of the development of C. vulgaris in the dark

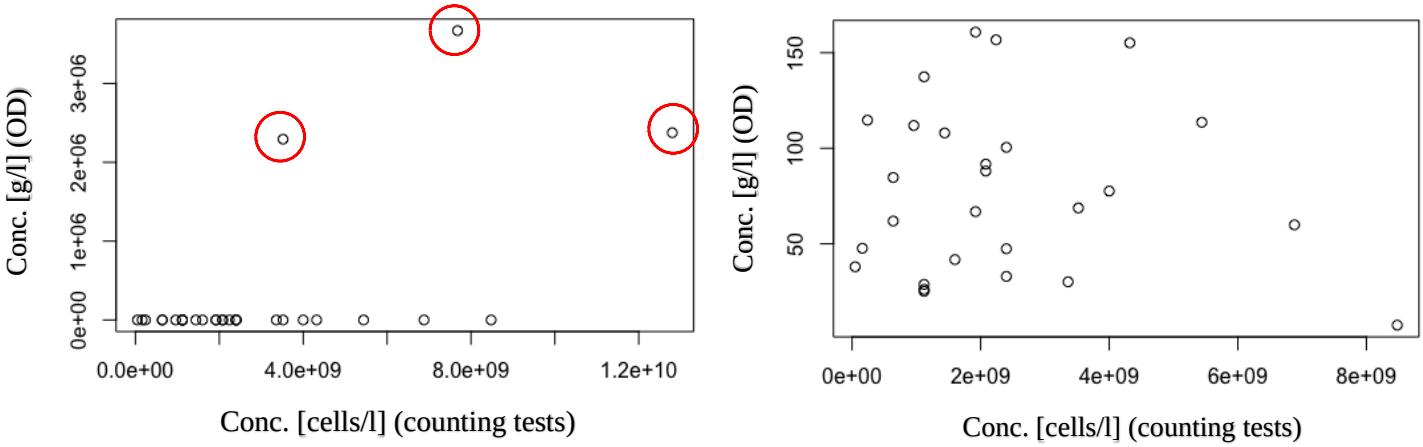


Figure 41*: Mass concentrations obtained by OD against cell concentrations obtained by counting tests of the development of C. vulgaris in C (control with microalgae in the HC and no roots no mycorrhizae in HC and RC) , S (control with microalgae in the HC and roots and mycorrhizae in the RC) and E (treatment with microalgae and mycorrhizae in the HC and roots and mycorrhizae in the RC) Petri dishes (R. irregularis MUCL 41833 and R. intraradices MUCL 49410) after 13 days of incubation in the dark. The second plot is the same as the first plot without the data of the 3 results that are circled in red.*

B. Experiment 1 with gelled hyphal compartment

Microscopic observations

As the results of this experiment are in the form of a description of what was observed under the microscope, a table (Table 18) was made to support what is described in this section. The table includes a first column that reflects the development of the microalga in the gelled compartment. These results are in the form of +'s. The other 3 columns show the fraction of big, average and small colonies of microalgae. This table is subjective, no results were calculated. It is an approximation of the reality and gives the observer's impression.

For this experience on gelled compartments, the microalgae had again developed best on the C Petri dishes, followed by the microalgae growing on the two sets of S Petri dishes (*R. aggregatum* MUCL 49408 and *R. intraradices* MUCL 49410). Microalgae on the E Petri dishes had the poorest development. Although there is still some diversity within the same group, clear tendencies are observable. In general, *S. dimorphus* grew better than *C. vulgaris*. Sometimes the difference is minimal, sometimes it is important. Another observable difference was that *C. vulgaris* seemed to have a slightly faster tendency to form big colonies than *S. dimorphus*. For a similar development, *C. vulgaris* had on average more large colonies. There was more heterogeneity in the development of *C. vulgaris* than in that of *S. dimorphus*; *S. dimorphus* had more colonies of the same size. In contrast, *C. vulgaris* sometimes still had many small colonies and already very large ones, too. Nevertheless, in general for a good development, more big colonies were observed, for a medium development more average colonies were observed and a bad development was usually coupled with a lot of small colonies.

The global results are similar to those obtained by the previous experiment with liquid microalgal compartments; there is a clear difference between the C group which has the best growth, except that this time there seems to be also a clear difference between the S and E groups.

What was also noted, is that, although care was taken to select hyphae that were near the surface and to apply the microalgae droplets close to them, microalgae were not everywhere in direct contact with AM fungal hyphae. When they were in contact with each other, hyphae end up diving into the medium, leaving the microalga behind, unable to further follow the fungi. In general, where microalgae are at the same depth as hyphae, they tend to follow them (Figure 43). Microalgae generally gather in a circle, except when they are in contact with the hypha; Cells follow each other in a more longitudinal way (Figure 43).

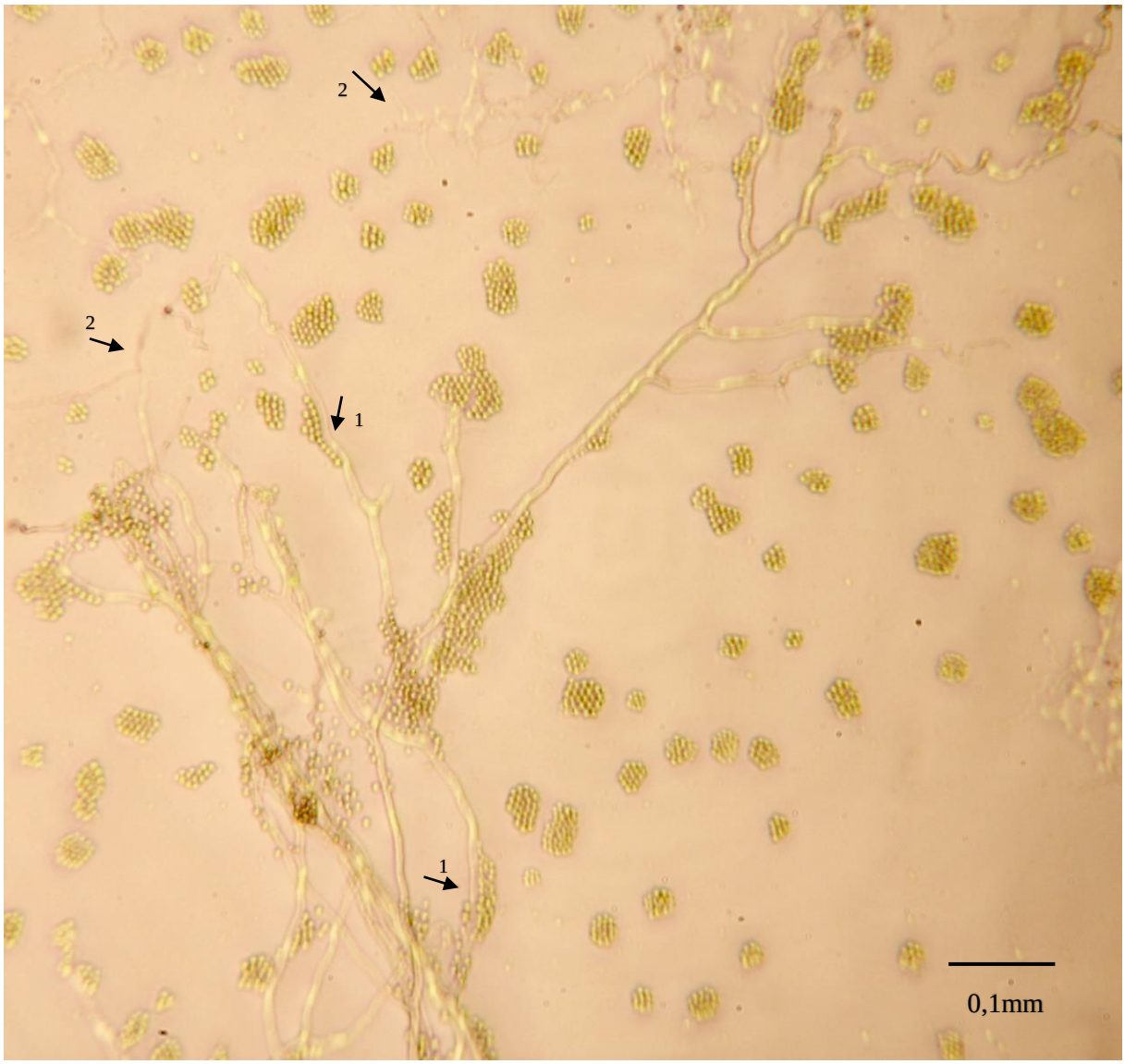


***Figure 42**: C. vulgaris and R. aggregatum MUCL 49408 hyphae on gelled MSR- bi-compartmentalized Petri dish of the E (Microalgae and AM fungal hyphae in the HC, roots and AM fungal hyphae in the RC) treatment after 14 days of incubation in the dark. The arrows (1) indicate microalgae following a hypha and the arrows (2) indicate diving hyphae.*

Observation of microalgae in contaminated Petri dishes enabled to see that their development was highly suppressed in the presence of contaminations.

If microalgae seemed to have difficulties to thrive next to mycorrhizae in this closed environment, mycorrhizae didn't seem to have difficulties to further grow. In contrary, they seemed to blossom. The hyphae expanded in the whole compartment, comparison of pictures taken on day 1 and day 14 allowed to discover plenty new hyphae and spores. Some microalgal spots where no hyphae could be seen on the surface on the pictures of day 1 were crossed by hyphae on the pictures of day 14.

As mentioned before and what can also be deducted from table 18, the microalgae did not develop the same way in each replica of the same group (Figure 44: The corresponding picture taken on day 1 of picture a can be found in appendix 5, Figure 45).

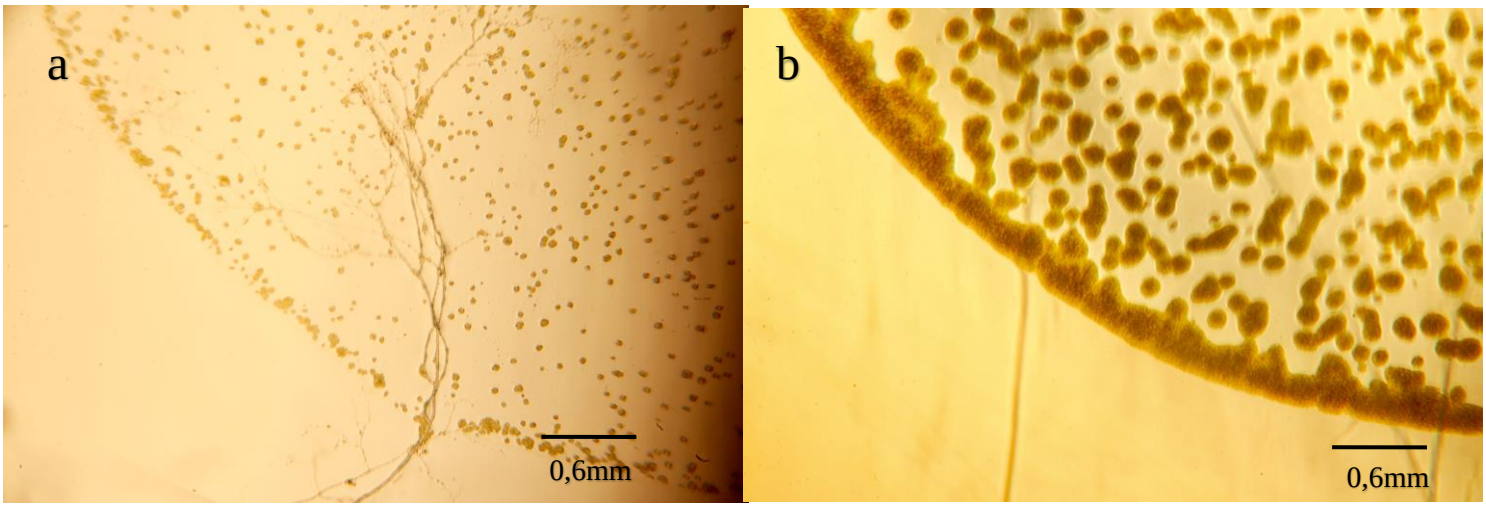


***Figure 44**: C. vulgaris on R. intraradices MUCL 49410 mycorrhized gelled MSR- HC on day 14. Picture a corresponds to the 4th replica of its group in table 18. Picture b corresponds to the 1th replica of its group in table 18.*

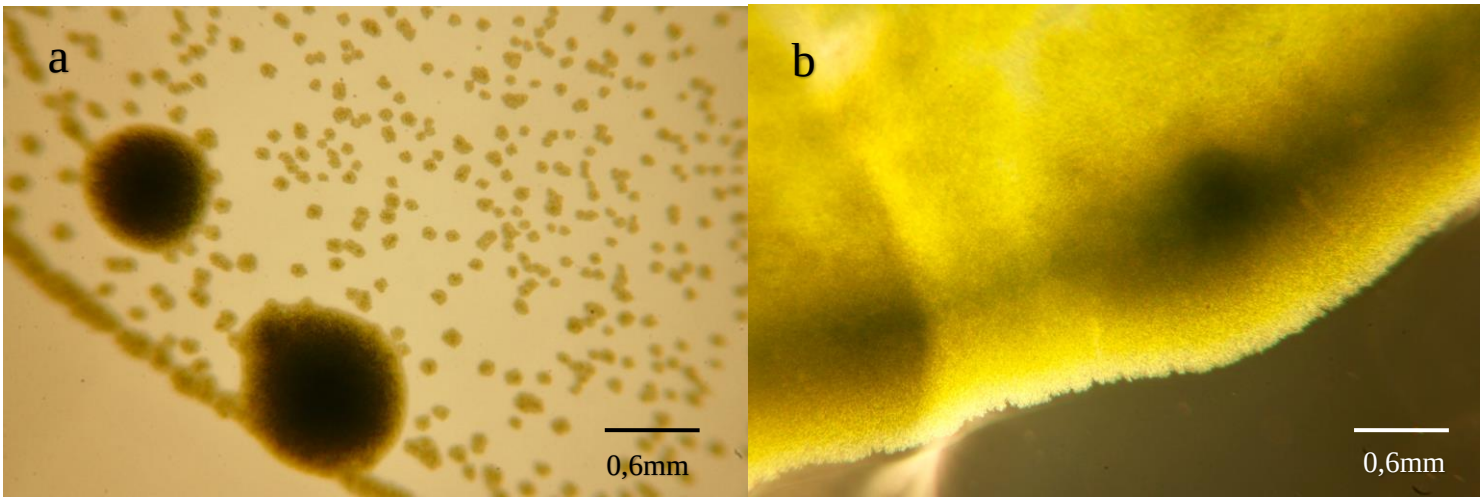


***Figure 43**: S. dimorphus on gelled MSR- HC of C (control) Petri dish on day 14. Picture a corresponds to the 2th replica of its group in table 18. Picture b corresponds to the 5th replica of its group in table 18.*

Table 18 *: Development and number of small, average-sized and big colonies of S. dimorphus and C. vulgaris in gelled MSR- HC of C (microalgae in the HC/ no roots no AM fungus in HC and RC) , S (microalgae in the HC/ roots and AM fungus in the RC) and E (microalgae and AM fungus in the HC/roots and AM fungus in the RC) of R. aggregatum MUCL 49408 and R. intraradices MUCL 49410 treatments after two weeks of incubation in the dark.*

			S. dimorphus				***C. vulgaris***			
			Devpt	**Small c.**	**Average c.**	**Big c.**	**Devpt**	**Small c.**	**Average c.**	**Big c.**
CTRL	C	1	+++++	0	0	1	++++	0	0	1
		2	+++++	0	0	1	++++	0	0	1
		3	+++	0.1	0.6	0.3	+++	0.20	0.65	0.15
		4	+++	0	0.2	0.8	+++	0	0.3	0.7
		5	++	0.2	0.5	0.3	++	0	0.5	0.5
49408	S	1	++++	0	0.25	0.75	+++	0.1	0.3	0.6
		2	+++	0.1	0.7	0.2	+++	0.1	0.4	0.5
		3	+++	0.1	0.7	0.2	+++	0.15	0.2	0.65
		4	+++	0.2	0.5	0.3	++	0	0.5	0.5
		5	++	0.6	0.4	0	++	0.1	0.55	0.35
	E	1	+	1	0	0	+	0.95	0.05	0
		2	++	0.9	0,1	0	++	0.9	0.1	0
		3	+++	0.8	0,2	0	++	0.75	0.25	0
		4	++	0.75	0,2	0.05	++	0.8	0.2	0
		5	++	0.8	0,2	0	++	0.8	0.2	0
49410	S	1	+++	0.1	0.8	0.1	+++	0.35	0.35	0.3
		2	+++	0.2	0.8	0	++	0.2	0.6	0.2
		3	++	0.3	0.7	0	++	0.3	0.7	0
		4	++++	0.05	0.9	0.05	+++	0.3	0.6	0.1
		5	++	0	0.9	0.1	++	0.2	0.7	0.1
	E	1	+++	0.30	0.50	0.20	+++	0.35	0.45	0.20
		2	++	0.75	0.25	0	++	0.85	0.15	0
		3	++	0.7	0.3	0	++	0.75	0.25	0
		4	++	0.85	0.15	0	++	0.9	0.1	0
		5	+	1	0	0	+	1	0	0

With Dvpt= Development, c.= colonies

***Table 19**: Results of phosphorus tests of the gelled MSR- HC of the C (microalgae in the HC/no roots no mycorrhizae in HC and RC) , S (microalgae in the HC and roots and mycorrhizae in the RC) and E (treatment with microalgae and mycorrhizae in the HC/ roots and mycorrhizae in the RC) of R. aggregatum MUCL 49408 and R. intraradices MUCL 49410 treatments after two weeks of incubation in the dark.*

		P [mg/kg]			
		1	**2**	**3**	**Mean**
CTRL	C	6.9	6.06	6.6	6.52
R. aggregatum MUCL 49408	S	5.16	5.44	5.55	5.38
	E	4.44	4.69	6.39	5.17
R. intraradices MUCL 49410	S	6.64	6.14	6.67	6.48
	E	2.74	2.68	3.32	2.91

Phosphorus tests

Phosphorus concentrations on three replicates of each treatment of the gelled HC at the end of the 13 days were measured. We observe the highest average phosphorus concentration for the C treatment (6.52 mg/kg), followed by the S treatments (6.48 mg/kg for *R. intraradices* MUCL 49410 and 5.38 mg/kg for *R. aggregatum* MUCL 49408) and the E treatments (5.17 mg/kg for *R. aggregatum* MUCL 49408 and 2.91 for *R. intraradices* MUCL 49410).

C. Experiment 2 with gelled hyphal compartment

For this experiment as well, a table (Table 20) was written to support the description of the observations under the microscope. The table contains the same columns as Table 18. The (subjective) scales are roughly the same, but the conditions in which the two experiments were realized were not quite the same. As a reminder, microalgae were added at lower initial concentrations in this experiment.

For this third experiment on the development of microalgae in the dark in the presence and absence of mycorrhizae, overall results were not very different from the results of the first two experiments. The main commonality is that there was a better development of the microalgae from the C and S controls than of the microalgae of the E treatments. The microalgae in the HC of the E treatments have never become very large. Those Petri dishes contained mainly small colonies. The replicates of the C treatment were more similar to each other in this experiment as compared to replicates of the C treatment in the previous experiments and as compared to the replicates of the S treatment of this experiment. However, the S treatment is composed of only 3 replicates; In one of the plates, the AM fungus grew in an unexpected way in the root compartment, entering the microalgae compartment. Microalgae had developed very well in this compartment. Figure 8 illustrates the hyphae growing towards *S. dimorphus* in that microalgal compartment. The E Petri dishes had again a boom of mycorrhizal growth in the hyphal compartment, it seems even more pronounced than in the previous experiment. Mycorrhizae that had just entered the microalgal compartment on day 1, had colonized the whole space after 2 weeks (Figure 47). Furthermore, an intense hyphal branching was observed (Figure 46).

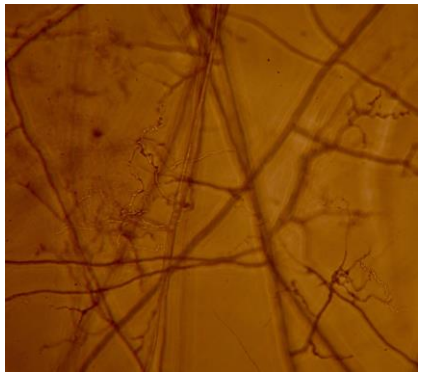


***Figure 45**: Intense hyphal branching of R. aggregatum MUCL 49408 in HC (MSR-medium) with microalgae*

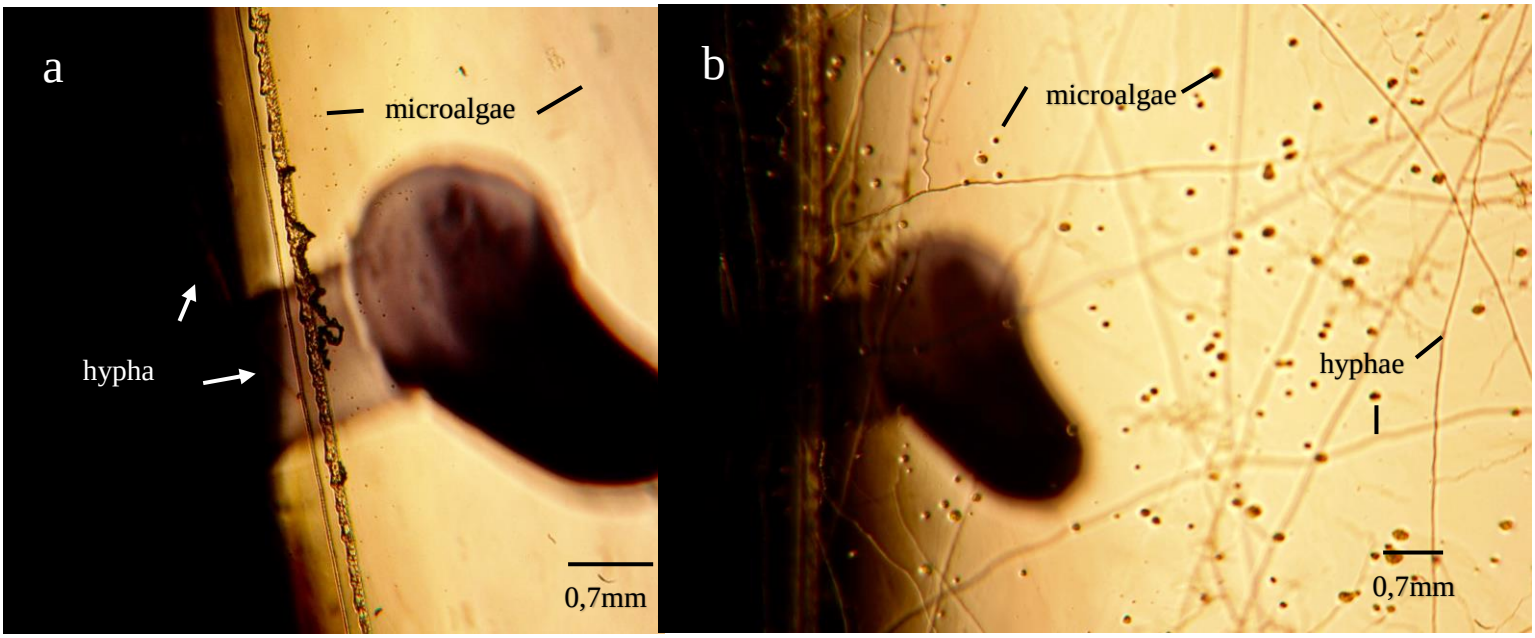


***Figure 46**: S. dimorphus on gelled R. intraradices MUCL 49410 mycorrhized MSR- HC of an E (microalgae and SM fungus in HC/ roots and AM fungus in RC) treatment on day 1 (a) and day 14 (b). The Pictures correspond to the 3th replica of its group in table 20.*

Taking a look at what happened in the root compartment showed that hyphae were very active in this compartment, too.

In the globality of the whole experience, *S. dimorphus* had, here again, developed better than *C. vulgaris*. The two microalgae behaved differently, too. For *C. vulgaris*, more small and large colonies were observed in comparison with *S. dimorphus*. The colonies of this latter were of a more homogeneous size. Figure 48 illustrates well on the one hand the better development of *S. dimorphus* and on the other hand their difference in colony size distribution.

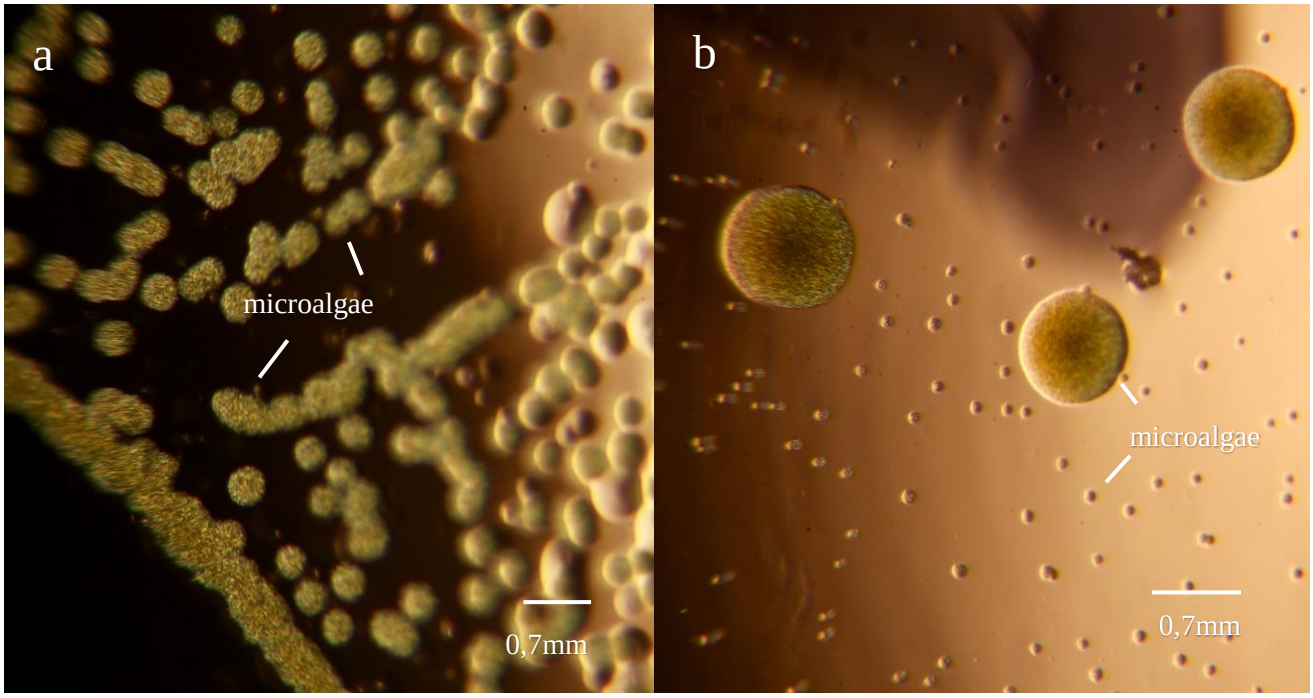


***Figure 47**: S. dimorphus (a) and C. vulgaris (b) on gelled MSR- HC of C (microalgae in HC/no roots no AM fungus in RC) treatment on day 14. The Pictures correspond to the 1th replica of their group in table 20.*

To conclude, an observation of the HC of the Petri dishes 2 weeks later, i. e. on day 30, revealed that the microalgae had not changed much since the observations on day 14. Also, in one E Petri dish, some roots had crossed over to the hyphal compartment. At some places along the roots, a flow of diffusing microalgae cells could be observed. The ends of the flow closest to the root diffused at a faster rate than the outer ends, where the microalgae were almost at a standstill (Figure 49).

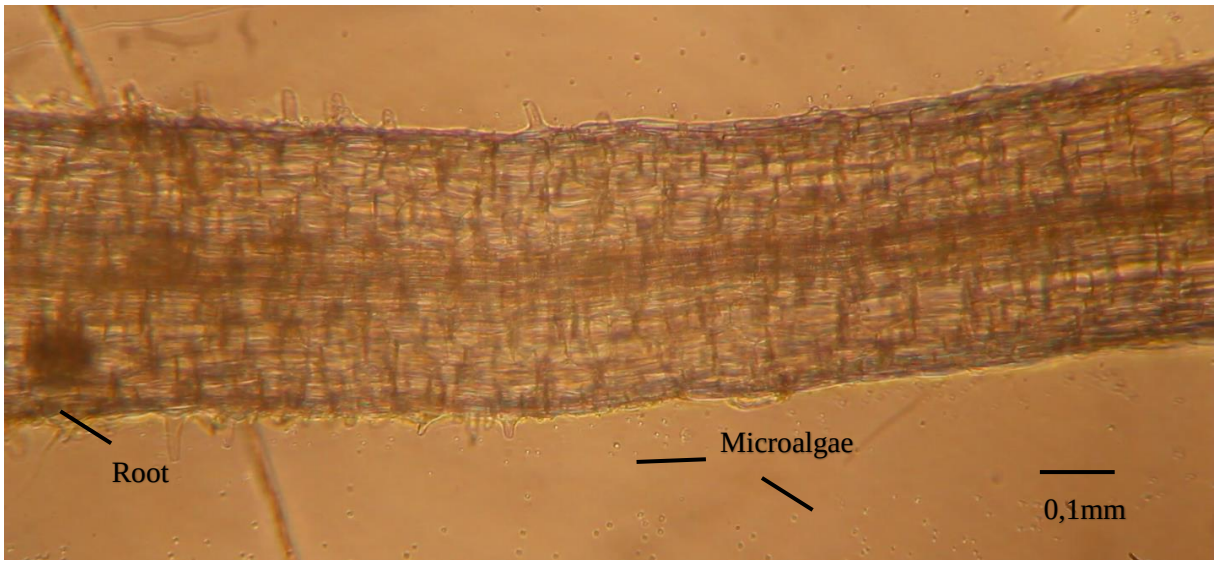


***Figure 48**: Flow of microalgal cells moving along a root that had crossed over in the HC of an E (microalgae and AM fungus in HC/ roots and AM fungus in RC) treatment after 30 days of incubation in the dark.*

Table 20: *Development and number of small, average-sized and big colonies of S. dimorphus and C. vulgaris in gelled MSR- HC of C (microalgae in the HC/no roots no mycorrhizae in HC and RC) , S (microalgae in the HC and roots and mycorrhizae in the RC) and E (treatment with microalgae and mycorrhizae in the HC/ roots and mycorrhizae in the RC) of R. intraradices MUCL 49410 treatments after two weeks of incubation in the dark.*

			S. dimorphus				*C. vulgaris*			
			Devpt	Small c.	Average c.	Big c.	Devpt	Small c.	Average c.	Big c.
CTRL	C	1	++++	0,15	0,6	0,25	++	0,6	0,15	0,25
		2	++++	0,25	0,55	0,2	+++	0,65	0,35	0
		3	++++	0,4	0,5	0,1	++	0,60	0,3	0
		4	++	0,6	0,4	0	+	0,6	0,3	0,1
49410	S	1	++++	0,1	0,45	0,45	+++	0,45	0,1	0,45
		2	++	0,65	0,35	0,1	++	0,65	0,35	0
		3	+	0,90	0,1	0	+	0,95	0,5	0
		4*	++++	0,1	0,35	0,55	+++	0,6	0,1	0,3
	E	1	+	1	0	0	+	1	0	0
		2	++	0,95	0,05	0	+	0,9	0,1	0
		3	+	0,95	0,05	0	++	0,95	0,05	0
		4	++	1	0	0	++	1	0	0
		5	++	0,95	0,05	0	++	1	0	0

With Devpt= Development, c.= colonies

**Petri dish that had a bad colonization of hyphae in the beginning and thus no mycorrhizae in the hyphal compartment, but it underwent a boom of colonization during the experience and some hyphae passed through the microalgal compartment when the petri dishes were observed. This Petri dish is not to be considered in the S treatment anymore.*

V. Discussion

I. Impact of the microalgal exudates on the germination of spores, hyphal branching and elongation of germinating hyphae

The findings suggest that the exudates of *S. dimorphus* cultivated in 3N-BBM medium don't have much impact on the germination of AM fungal spores. All the treatments induced a good germination rate. About the timing at which the spores germinated, only treatment 2.5% of the strain *R.intraracides* MUCL 49410 induced a later germination, relative to the control and the 0.25% treatment of the same strain, but not relative to *R.aggregatum MUCL* 49408 treatments and the second control.

Results of hyphal elongation and ramification were quite different for *R.intraracides* MUCL 49410 than for *R.irregularis MUCL* 41833 and *R.aggregatum MUCL* 49408. For the two latter strains, hyphal growth seems to go with hyphal ramification as the treatments who induced an increased hyphal elongation were the same as the ones who induced a higher ramification of the hyphae. For both strains, the treatment 25% and for *R.irregularis MUCL* 41833 the treatment 2.5% as well, had a significant impact on both hyphal elongation and branching. For *R.intraracides* MUCL 49410, it was also the treatment 2.5% who induced a higher hyphal elongation, but it was not coupled with an enhanced ramification. The surprising part is that the treatment 25% did not induce an enhanced hyphal growth (and ramification).

Considering the results of *R. intraradices* MUCL 49410, it could be hypothesised that one or more molecules exuded by *S. dimorphus* have a stimulating effect on hyphal growth from a certain concentration up to a certain concentration, where that or these molecules have no effect on hyphal growth any more. One could make a similar analogy to glucose, which has a stimulating effect at low concentration and an inhibitory effect at higher concentration, according to Siqueira *et al.* (1985) on spore germination and experienced in this work, on the growth of *S. dimorphus*. However, the treatment 25% although not significantly different, induced on average a twice as high hyphal elongation as compared to the control and the treatment 0.25% of this strain. If the treatment 25% did not have the same stimulating effect on hyphal elongation as treatment 2.5%, it did not have an inhibitory effect either. It would therefore be interesting to conduct tests at even higher concentrations to see whether higher concentrations inhibit hyphal growth or not and if hyphal growth of the two other strains is also negatively affected by higher concentrations of exudates.

It is possible that one hypothesis may be tested for one strain and not another, not all strains react in the same way. But a hypothesis that would support the results obtained for the three strains would be to say that all strains have different sensibilities to microalgae exudates and that *R. irregularis* MUCL 41833 and *R. aggregatum* MUCL 49408 have a higher threshold of inhibitory effect than *R. intraradices* MUCL 49410. We can already see that they have different stimulation thresholds (i.e. the 2.5% treatment already induces hyphal growth stimulation of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410, but it is only from the 25% treatment that the exudates have also stimulating effects on hyphal growth of *R. aggregatum* MUCL 49408).

In all cases, this experiment showed that exudates of *S. dimorphus* grown in a 3N-BBM medium, added at a certain concentration in a solid MSR- medium, have a stimulating effect on the hyphal elongation and branching of germinated spores of *R. intraradices* MUCL 49410, *R. irregularis* MUCL 41833 and *R. aggregatum* MUCL 49408. Furthermore, it allowed to see that hyphal elongation of *R. intraradices* MUCL 49410 velocities with increasing concentrations of *S. dimorphus* exudates were constant over a time period of 7 days and that the effects of *S. dimorphus* exudates on hyphal growth were already significant after 2 days of hyphal elongation.

However, as the culture medium of *S. dimorphus* supernatants (3N-BBM) was not the same as the one used in the Petri dishes (MSR-) and the amount of supernatants added to each treatment was not equal, it seemed opportune to take a closer look at its composition. There was a concern that elements from the 3N-BBM medium act on spore germination and hyphal growth. The mineral composition of a medium is important for the plant-AM fungi association.

Plants allocate more biomass in structures allowing them to acquire the most limiting elements they need. If the most limiting elements are light and carbon dioxide, the plant will tend to produce more vegetative apparatus. If, on the contrary, limitations come from underground (like a phosphorus limitation), the plant will tend to produce more roots. So, this functional equilibrium model is at the basis of the mycorrhizal association. If the plant needs the association for limiting resources, it will put energy into it. If not, it will concentrate its energy into something else. Two crucial elements at the basis of mycorrhizal interaction are nitrogen and phosphorus. Johnson *et al.* (2010) developed a trade balance model that predicts mycorrhizal association regarding to the C-N-P (carbon-nitrogen-phosphorus) stoichiometry (Figure 50). This model has numerous limitations as it goes without saying that a tremendous number of other factors may come into play and mycorrhizae have more commodities to carbon than these two mineral ions, but in this context of controlled environment it permits to recognize the importance of nitrogen and phosphorus concentrations in the culture medium.

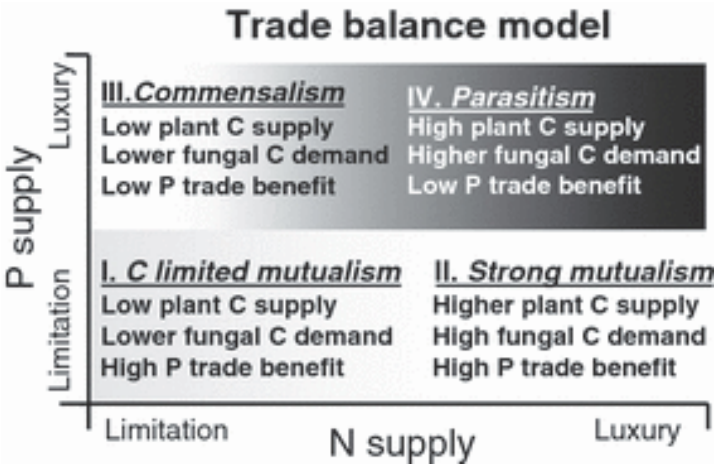


Figure 49: *Trade balance model (Johnson, 2010)*

In this trade balance model, the mycorrhiza trades phosphorus against carbon. As three quarter of nitrogen in the leaf is invested in chloroplasts in order to produce carbon, a high availability of nitrogen permits to produce carbon through photosynthesis. In the case of a P-limitation, carbon is exchanged against P and a high N availability enhances photosynthesis, which boosts the carbon production and trade for phosphorus. This stoichiometry induces the best mycorrhizal association. A P- and N-limitation will also support the beneficial interaction, but the C for P trade will be limited by limited N availability. If the P and N are both abundant, fungal C demand will only be limited by the carbon and thus the mycorrhizal demand for C may increase in such a way that one may speak of parasitism. An abundant P and limited N availability will bring plant fungus in

competition for nitrogen and carbon and C transfer to the mycorrhiza is going to be limited by N. As one might see, a limited phosphorus concentration is always beneficial for the association. Phosphorus is a mycorrhizal crucial element of exchange for carbon. On the other hand, the most beneficial concentration of nitrogen depends on the availability of phosphorus (Johnson, 2010).
In the mycology laboratory, low concentrations of phosphorus and average concentrations of nitrogen are used in the culture media in order to promote the association. When concentrations of P and N in the 3N-BBM medium and the MSR- medium are brought aside, one can see that phosphorus and nitrogen concentration are respectively more than 50 and 2 times higher in the 3N-BBM medium than in the MSR- medium (Table 21).

Table 21*: Concentrations of P and N in 3 N-BBM and MSR- media*

	3N-BBM	MSR-
P [mM]	1,72	0,03
N [mM]	8,82	3,98

As the important stoichiometry of these elements has been highlighted above, these differences are not to be neglected. However, in the course of the ALGOTECH project, it was planned to induce nitrogen hunger at the end of the cultivation in order to promote the production of lipids in microalgae. When that happens, there is no need to worry about having a concentration of mineral nitrogen that would disturb the association. But the supernatants that were used for experience 1 did not undergo such depletion yet. However, Siqueira *et al.* (1985) affirm nitrogen and other salts don't affect spore germination. In contrast, they say phosphorus has an inhibitory effect. About the phosphorus, measurements were realized in order to see how much of the initial concentration was left after microalgal culture and filtration. They were realized with an inductively coupled plasma spectrometer (ICAP 6500 duo view) form Thermo Scientific. A filtration was required beforehand. The measurement was carried out at 4 different wavelengths (177.495, 178.284, 213.618 and 214.924) with 213.618 being the most sensitive. The supernatant samples were diluted 10 times before measurement. Table 22 assembles concentrations of P in both 3N-BBM and MSR- media, *S. dimorphus* supernatants and the average concentrations of period 2006-2016 in rotating crops and market gardens in Wallonia.

Table 22 *: Concentrations of P in MSR- and 3N-BBM media, S. dimorphus supernatants and the average concentrations of period 2006-2016 in rotating crops and market gardens in Wallonia*

		[P] (ppm)
MSR-		0.93
3N-BBM		53.5
S.dimorphus	Sample 1	63.5
supernatants	Sample 2	33
	Sample 3	48.5
Rotating crops (2006-2016 Walloon)*		81.4
Market gardens (2006-2016 Walloon)*		136.1

*http://www.requaconsult.requasud.be/requaconsult_sol, consulted on 25/5/19

As we can see, phosphorus concentrations in the supernatants are still very high. Phosphorus could have contributed to the effects observed by the supernatants on spores and hyphae.

However, Hepper (1983) tested effects of different concentrations of phosphate on germination of spores and hyphal elongation of *Glomus caledonium*. Germination was affected at levels above 30 mM and hyphal extension was affected at levels above 10 mM. If *R. irregularis* MUCL 41833, *R intraradices* MUCL 49410 and *R. aggregatum* MUCL 49408 are affected in a similar way to phosphorus concentrations, even the higher phosphate levels present in 3N-BBM exudates would not affect their germination and elongation.

Given the applied objective of the project, it is also interesting to note that the levels of phosphorus present in Walloon soils are also very important. In this context, what is the room for manoeuvre to promote interaction between the AM fungus and plants of agronomic interest, if high phosphorus concentrations are an obstacle for AM fungus-plant associations? The answer may lie in the use of interaction stimulating effectors and it is this approach that is developed through the search for organic molecules (flavonoids, strigolactones, or others).
Furthermore, the high phosphorus concentration in the Walloon soils are mostly due to the big amount of fertilizers that are applied, pig manure for example. A legislation that regulates the amount applied on soils such as nitrogen does not exist for phosphorus (Rennesson, 2018). If one stops to apply phosphorus-rich fertilizers, the phosphorus concentration will drastically decrease. As only a small fraction of the phosphorus is assimilable by the plant, plus the phosphorus is poorly mobile, exchanging a phosphorus-rich fertilizer for a biofertilizer might, when a new equilibrium has established, have as a result a decrease of phosphorus in soils and a real need for plants to associate with mycorrhizae in order to get it.

The second set of experiments with exudates grown in MSR(+ and -) medium made it possible to dispel doubts about the 3N-BBM medium, to compare the effects of exudates from one microalgal strain (*C. vulgaris*) grown in light and darkness on spore germination, hyphal elongation and branching of a strain of AM fungus (*R. irregularis* MUCL 41833) and to compare the effects of exudates of two strains (*C. vulgaris* and *S. dimorphus*) grown in darkness on spore germination, hyphal elongation and branching of an AM fungus strain (*R. irregularis* MUCL 41833).

It is clear that spore germination caused some problems during this experiment. In the first trial, half of the treatments had a germination rate below 50%. The treatments 2.5% had the poorest germination rate of all (5.6%, 0% and 22%). Not much can be deduced from the results of treatment 2.5%, except that perhaps at this concentration exudates have an inhibitory effect on spore germination. However, the repetition of the experiment discredited this hypothesis because the control treatment induced a germination rate of 0% and *S. dimorphus* exudates cultivated in the dark of more than 50%. As it would seem that the exudates are not at the root of low spore germination, there are other factors at play.

The problem of considerable and unexplained variability in germination between populations of spores has been brought forward by many authors (Daniels & Graham, 1976; D'Souza *et al*., 2013; Gerdemann & Trappe, 1974; Godfrey, 1957; Hepper & Smith, 1976; Tommerup, 1983). Reasons can be multiple and there are a few we can put the finger on like too old or too young mycelial network, nutritional imbalance, structural barriers, cultural history and selfinhibitors but some are ununderstood. Factors influencing low germination rates are temperature, pH, moisture, light radiation (better in the dark), aeration, concentration of inorganic ions (Siqueira et al., 1985). However, in the context of this work, these parameters were the same for all the spores during the whole period manipulations were conducted, which suggests they are not the cause. In the words of Siqueira *et al.* (1985), nutritional conditions that inhibit spore

germination are cited more often than stimulatory ones. Carbohydrates, organic acids, and amino acids have been found to suppress spore germination (Hepper, 1979; Siqueira *et al.*, 1985). Thiamine, dialysates, filtrates, root exudates, soil volatile compounds and small amounts of glucose have been found to stimulate spore germination (Hepper and Smith, 1976; Daniels and Graham, 1976; Siqueira *et al.*, 1985).

In the context of this thesis work, spores were considered as non-germinating spores after 3 weeks, but according to D'Souza *et al*. (2013), the time required for spore germination of different genera may range from 2 to 90 days. However, Tommerup (1983), who tested spore dormancy on different species and with varied conditions of storage (at different temperatures and periods of times) found that germination occurred with close synchrony. For example, spores of four strains that had been stored during 5 to 7 months in a soil at 22°C, all germinated between 3 to 5 (for two strains) or 10 to 12 (for the two other strains) days. Therefore, as a major part of the spores (who all comes from the same subculture) had germinated within the two weeks, it seems unlikely that the other spores would still germinate after three weeks. Moreover, D'Souza *et al*. (2013) obtained, in an experiment testing the effects of sucrose on germinating spores, the maximum germination rate (of 90%) of *R. irregularis* after 18 days for their control treatment. *R. irregularis* MUCL 41833 caused germination problems in a number of other manipulations during that period in the Mycology laboratory, further research is needed to understand why.
Perhaps explanations should also be sought in a science that is less obvious, such as the effect that the moon's cycle has on living organisms. But although this science is still poorly understood and could therefore be full of undisclosed secrets, the few studies that focus on the effect of the moon's cycle on fungi have failed to demonstrate any influence or have subsequently proved to be inconsistent (Egli *et al*., 2011). Whereas Egli *et al.* (2011) suggest that the moon cycle has no effect on the growth of mycorrhizae, their research is based on the fruiting rate alone. Richter (2006) further states that for most fungi, there does not seem to be a significant correlation between the quantities of fungi found and the phases of the moon, but according to him, we should not assert the influence of the moon, nor exclude it in principle; The fact that we do not understand does not mean that it does not exist.

About the timing of spore germination, exudates of *C. vulgaris* cultivated in the dark and in the light and of *S. dimorphus* cultivated in the dark did not seem to impact it. Contrariwise, at the concentration of 25% in a solid MSR+ (1g/L glucose) medium, the exudates all stimulate hyphal growth and ramification of germinated spores of *R. irregularis* MUCL 41833. In addition, it seems that *C. vulgaris* produces different molecules or the same molecules in different quantities that act on hyphal branching depending on whether it is autotrophically or heterotrophically grown. Exudates from autotrophic and heterotrophic cultures induced the same elongation of hyphae, but those issued from autotrophic culture induced in comparison to those issued from heterotrophic culture a higher hyphal branching. *C. vulgaris* and *S. dimorphus* in the dark induced the same elongation and branching of hyphae. It is assumed that both strains release the same type of molecules and in sufficiently similar quantities to have the same effect on germinated spores. However, microalgae do not grow as fast. Here the cultures had been diluted such as to have as many microalgae cells in all supernatants before filtration. Moreover, *S. dimorphus* revealed to grow twice as fast in autotrophic conditions than in heterotrophic conditions. Contrariwise, *C. vulgaris* revealed to grow as fast in both carbon acquisition regimes.

C. vulgaris and *S. dimorphus* exudates, grown in 3N-BBM and MSR (+ and-) medium have shown stimulating effects on the growth and branching of hyphae. It would now be interesting

to identify these stimulating molecules. Khan *et al.* (2009) evoke that many seaweed and seaweed-derived products have been widely used as amendments in crops due to a number of plant growth-stimulating compounds, but the their biostimulatory potential has not been fully exploited due to the lack of scientific data on growth factors and their mode of action in affecting plant growth. The same applies to mycorrhizal growth-stimulating compounds in algae. Many identified molecules have already demonstrated stimulating effects on hyphae growth. These molecules might be the ones inducing stimulatory effects on AM fungi in microalgal extracts. We are talking about flavonoids, strigolactones, mannitol, carrageenan, sugar alcohols such as xylitol, sorbitol, alginate oligosaccharides, polyamines, quercetin, myricetin and others (Akiyama and Hayashi, 2006; Khan *et al*., 2009; Kuwada *et al.*, 2005; Ishii *et al*. 2000). Using supernatant biochemical analysis methods (HPLC/MS) and extraction methods, it would be possible to identify and then isolate effectors that promote interaction and allow mycorrhizae to develop all their skills in the service of cultivated plants, including improving water and mineral nutrition, the structural and environmental state of the soil, the establishment of communication networks and crop protection.

II. Effects of microalgal exudates encapsulated with mycorrhizal spores on germination of spores and germinating hyphal elongation

This first experiment has given us interesting results about the stimulation of AM fungal hyphae by microalgae exudates. A next step with a perspective on the valuation of these exudates in agriculture was to test a method of application. The microalgae exudates were encapsulated with spores in microbeads. Two hyphae elongation measurements (total hyphae lengths emerging from a bead and total hyphae lengths of a Petri dish containing 5 beads) showed that exudates added at a concentration of 10% stimulate hyphae elongation, as compared to a concentration of 0% or 100%. Thus, they stimulate the growth of hyphae at a certain concentration. However, at a certain higher concentration, this stimulation no longer takes place. The growth of hyphae is not inhibited neither, because the exudates added to the concentration of 100 % induce the same hyphal growth as the control. The findings of this second experiment support the assumption that was made in regard to the results of the first experiment, which was to say that microalgae exudates have a stimulating effect from a concentration to a concentration where they have no effect.
It would be interesting to measure the colonization rate, as this is ultimately the first step in the symbiosis of the AM fungi-plant. Improved hyphae growth is useless if colonization does not follow behind. The time schedule did not permit to collect roots for measuring their colonization in the context of this master thesis. Mycorrhizal colonization will be assessed in perspective, by coloration when cultures are at least at 60 days old.

These two experiments have been promising for the use of microalgae supernatants as biofertilizer/pesticides. Encapsulating them with spores and applying them in the field could allow intensive elongation and branching of the spores, once germinated, which would result in AM fungal-plant associations beneficial to the plant. However, this gives resonance for two thoughts. The first reflection concerns the addition of exogenous spores. The intelligence of introducing a foreign strain can be questioned. Many authors evoked that intensive agriculture negatively affects spore diversity and quantity. However, their studies were almost ultimately held on the top soil layers (Oehl *et al*., 2005). Oehl *et al*. (2005) have shown that fields subjected to intensive agriculture still contain a lot of spores in deep soil layers. The species persist by

mean of a ‘gene bank’, which could restore the AM fungus community when switching to a more extensive farming. Introducing new spores might not be necessary. The second queries the need to use the exudates of microalgae to stimulate plant-fungus interaction. In undisturbed environments, mycorrhizae find their way to the plant. There is no shortage of plant/mycorrhizae symbioses. Wouldn't adding exudates perturbate the plant-fungus balance? Shouldn't we just stop disrupting life underground and let the mushrooms do their magic? The response might lay in the use of microalgae exudates as an intermediate solution to pass from an intensive agriculture to a more extensive one.

III. Effect of AM fungal hyphae exudates on the microalgae growth

The preliminary tests made it possible to set up a protocol of experimentation. During these assays, it has also been shown that streptomycin inhibits the growth of *S. dimorphus* in a very poor way. Experiments on several generations of microalgae may provide a more important desired inhibitory effect. There was also demonstrated that *S. dimorphus* could be grown in heterotrophic and mixotrophic cultures, which was not known from the scientific literature. The cultures were carried out on petri dishes with MSR+ solid medium (glucose 1g/L and sucrose 10g/L) and in MSR+ liquid medium (glucose 1g/L and sucrose 10g/L), in the dark (heterotrophic culture) and in the light (mixotrophic culture). These cultures also allowed to see that *S. dimorphus* developed better with glucose 1g/L than with sucrose 10g/L. There was also revealed that at a concentration of 10g/L, glucose has an inhibitory effect on *S. dimorphus*. The cultures initiated in the context of this work also suggest that *S. dimorphus* has a better development in heterotrophic conditions than in autotrophic conditions, unlike *C. vulgaris* which has a similar development in heterotrophic and autotrophic cultures. These findings about *C. vulgaris* and *S. dimorphus* cultures are in line with the words of Merah *et al*. (2014), that say that heterotrophic and autotrophic growth rates of *C. vulgaris* are comparable and Richmond (2003) that say that *C. vulgaris* is one of the rare microalgae for which heterotrophic and autotrophic growth rates are comparable.

About the experiment with a liquid hyphal compartment, microscopic observation showed that the C (control with microalgae in the HC and nothing in the RC) treatments were greener than the other two S (control with microalgae in the HC and roots and AM fungi in the RC) and E (microalgae and AM fungi in the HC and roots and AM fungi in the RC) treatments, particularly 3 of the 6 replicates. It allowed to see that hyphae clustered at the beginning of the compartment; However, since the medium is liquid, the nutrients/sugars diffuse freely throughout the compartment, making it possible to exchange nutrients/sugars throughout the volume. These clusters were surrounded by microalgae. It suggests that microalgae seek contact with mycorrhizae. Furthermore, the heterogeneity of microalgae throughout the liquid and their accumulation along the walls was brought forward.

The OD measurements confirmed what was observed under the microscope: for the two strains (*R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410) a higher absorbance was observed for the microalgae culture in the HC of the C treatment in comparison with the S and E treatments, which were not different from each other.
Furthermore, there was detected that the highest rate of absorbance was at 680 nm. Readings at wavelengths 550 nm (who had been done before for *C. vulgaris*) and 680 nm allowed to see the results obtained by both wavelengths were highly correlated. Fixed wavelengths measurements

can thus be taken with those two wavelengths. This outcome is supported by Goswami's (2011) article which carried out OD measurements at wavelength 680 nm for *S. dimorphus* and *S. quadricauda*.

The assessment of *C. vulgaris* growth by mean of a Fuchs-Rosenthal cell gave the same results as the two other methods. In other words, after 13 days, a higher concentration of microalga was assessed for treatment C, as compared with the concentrations of the control S and treatments E. This method gave us also a glimpse of what was going on sooner than after 13 days. For both strains, it is onwards day 11 that the treatment C differs from one of the other treatments (from treatment S for *R. irregulairs* MUCL 41833 and treatment E for *R. intraradices* MUCL 49410). After 7 and 9 days of incubation, no differences were perceptible between the three treatments. Intermediate measurements also showed that the microalga was in its exponential growth phase because the differences in concentration between day 11 and day 13 were much greater than the differences in concentration between day 9 and day 11. Surprisingly, most of the concentrations obtained by mean of the counting tests were below the concentration applied initially. However, it is important to consider the standard deviation of the initial concentration. In addition, the accumulation of microalgae along the walls and their deposition on the ground could explain this decrease in concentration.

The results of this liquid compartment experiment are convergent towards the same assumption: There is a higher development of *C. vulgaris* in a HC without AM fungus, with neither root nor AM fungus in the root compartment, than in a compartment with or without AM fungus in HC, with roots and AM fungus in the RC. This is surprising since the only thing that distinguishes the controls C from the controls S is that their root compartment is free of mycorrhized roots. The hyphal compartment in which the microalga is placed is similar, emptied of mycorrhizae. The control S takes part of the experiment to rule out the possibility of volatile trading from one compartment to the other. If the results are taken as they are, this would mean that there is a volatile exchange between the mycorrhized roots of one compartment and the microalgae of the other compartment, that is harmful to the development of the latter. This hypothesis seems doubtful. Furthermore, a non-parametric correlation test demonstrated a weak correlation between the results obtained by OD and those obtained by counting cell at the end of the 13 days. The credibility of these results is thus questioned.

A positive aspect of this experiment with liquid HC, is the different possibilities to asses microalgal growth. That also allows to compare the methods. By comparing the results of different methods as a whole, it seems that they all go in the same direction. However, a statistical comparison of the data that could be compared revealed that there was little consistency in the results. A first identified problem is that the remaining final volumes were different from one replicate to the other. The volume decrease was random within the replicates, so not enough careful handling seems to be at the root of that problem. Working with liquid in Petri dishes requires to be extremely careful. The Petri dishes were subject to a high contamination rate. Moreover, working in the dark did not provide the best framework for very meticulous handling. It was also an obstacle for telling if microalgae were uniformly dispersed into the liquid before sampling to assess growth by mean of a Fuchs-Rosenthal cell. Although the liquid compartment was homogenized with a tip before sampling, errors related to the spatial distribution of algae throughout the medium have most probably occurred.
This experiment, without being repeated, did not allow a clear conclusion to be drawn. That was the reason why a new experiment was carried out, on solid medium.

A first experiment with microalgae placed on a solid MSR- medium in the hyphal compartment revealed that *C. vulgaris* and *S. dimorphus* had grown best in the C treatment, followed by the S treatment and finally the E treatment. There was a considerable diversity within the replicates, but trends were clearly observable. The experiment also showed that *S. dimorphus* grew better than *C. vulgaris* in the operating conditions. Moreover, their divergences in colonies forming were brought to light. Colonies of *C. vulgaris* are more heterogeneous in size, with more big and small colonies than *S. dimorphus* who had on average more colonies of the same size. Lastly, this experiment opened eyes to the boom of AM fungal growth in the hyphal compartment in the presence of microalgae.
Although all these hypotheses, which are based solely on observation, cannot be verified by statistics and do not allow undisputed conclusions to be drawn, they do open up prospects suggesting the development of microalgae being less favorable in the presence of hyphae in a dark controlled sugar-free environment with limited elements, while AM fungi seem to prosper in this same environment (having access to the root compartment with roots and sugars) in the presence of microalgae.
Why microalgae had a better grow in the C controls than in S controls remains misunderstood. But a hypothesis of the poorer results of the E treatment in comparison to the other two treatments, is that microalgae lack crucial mineral elements for their development, such as nitrogen and phosphorus, in the presence of hyphae. Hyphae could have absorbed all or a big part of the nutrients in vicinity of microalgae. Microalgae are not mobile; They have no flagella and the medium is gelled, they cannot move towards a place that is not depleted in mineral ions.

A lot of experiments point to the fact that algae growth is strongly linked to concentration of N and P in the environment. Several authors speak of a threshold effect. The threshold effect is a principle where under a certain concentration of the most limiting nutrient, algal growth is affected (Kunikane *et al.*, 1984). Kunikane *et al.* (1984) evoke that the N and P limitations are a multiplicative effect for *S. dimorphus*. In other words, the algal growth is dependent on the concentrations of all suboptimal nutrients which might be exerting influence simultaneously. They think a definite boundary of N-limiting and P-limiting does not exist and that none of the N and P concentrations in the medium and algal N and P contents solely regulates the growth of *S. dimorphus* in most of the ranges of N-P ratio. Besides, they suggest a much higher phosphorus concentration compared to the amount of nitrogen is necessary for *S. dimorphus* to maintain a high growth rate. Consequently, phosphorus measurements of the hyphal compartments were held at the end of the experiment.

The C treatment, S treatments and E treatment of strain *R. aggregatum* MUCL 49408 have pretty similar phosphorus concentrations, the E treatment of *R. intraradices* MUCL 49410 is lower. However, phosphorus is absorbed by hyphae and microalgae. In the close vicinity of these organisms, we hypothesize that P concentration must be very low and that it should gradually increases as we move away from hyphae (and microalgae). A total phosphorus concentration in the compartment does therefore not necessarily reflect the concentration available around the microalgae when they were applied, since they are applied near hyphae. Nielsen *et al.* (2002) who made experiments on the phosphorus uptake by *G. intraradices* in HC, evoked that after 37 hours of phosphorus added (1.25 µmol P per Petri dish), the HC became progressively P depleted. The depletion occurs close to hyphae and spreads further ahead. Bidirectional transport of P from both HC and RC was observed. Moreover, they discovered that the amount of transported P was poorly correlated with extraradical hyphal length, but highly correlated with the number of hyphae crossing the barrier separating the compartments. Thus, we dare to think the decrease in P and certainly also N that has not been

measured might be the reason of the poor development of the algae in the hyphal compartments with hyphae.

Furthermore, microalgae were suppressed in contaminated hyphal compartments: This goes with the hypothesis of limiting mineral elements in the microalgae compartment, because in a competitive environment, microalgae have more difficulty to develop.

A positive point of this experiment was the lower rate of contaminated Petri dishes. However, some aspects of the protocol were fallible and that is why a new experiment was launched.
First, as explained just above, microalgae could have lacked certain mineral ions for a good development in the presence of hyphae. Three weeks had passed between the moment the medium was poured in the hyphal compartment and the moment the microalgae had been applied on it. Mycorrhizae could have sampled most of the mineral ions in this compartment and brought it to the plants. In the second experiment, microalgae were applied right after solidification of the MSR- medium. Secondly, as mycorrhizae tend to dive into the medium and that microalgae are applied to the surface, microalgae were often not in direct contact with mycorrhizae. In the second experiment with gelled HC, the gelled medium was poured even thinner than in the previous one. Microalgae were applied in a line rather than in droplets on the freshly poured medium at the frontier between new medium where no hyphae had passed yet and the older medium slope, full of hyphae. The idea was that hyphae pass through the microalgal line, suggesting the microalgae to be in a place where there are enough mineral ions left and where they could receive sugars from mycorrhizae.

Fort his last experiment, the results have been repeated. Treatment C once again induced the best development of microalgae, followed by treatment S and finally treatment E. *S. dimorphus* also grew better than *C. vulgaris* and the dissimilarities in the distribution of colonies of different sizes were again theorized. One might think that for this experiment too, the microalgae of the E treatment lacked N and P. Algae were applied to an environment still rich in mineral elements, but they take time to develop. In two weeks, the hyphae, which colonized a large part of the HC, could have depleted the medium of N and P around the microalgae. This hypothesis does not answer the question of why microalgae of the C control grow better than those of the S control, nor the question of how microalgae of both treatments C and S obtain carbon. They are in a sugar-free environment unable to photosynthesize because they are in the dark. The only source of carbon they could have access to is the one that constitutes the gelling agent of the medium, Phytagel^{TM}. It is produced from a bacterial substrate composed of glucuronic acid, rhamnose, and glucose, that produces a high-strength gel (https://www.biobasic.com/phytagel, consulted on 22/05/19). However, no gelling agent was added in the liquid HC of the first experiment.

Furthermore, the inconsistency between the results of the preliminary assay (Interaction between AM fungal hyphae and microalgae in heterotrophic conditions on solid medium) in which microalgae had grown less in the absence than in the presence of mycorrhizae in the dark and the results of the three experiments here remains misunderstood. Two major differences can be identified between the assay carried out previously and the experiments. The first is that, for the assay, the microalgae were applied to a hyphal compartment that had been containing hyphae for 2 months, unlike the experiments which were 0 to 3 weeks. The second difference is that there were roots in the hyphal compartment of the assay. This difference suggests that roots inhibit the growth of microalgae and that in the presence of mycorrhizae, this inhibition is lessened.

To better understand what is really going on it would be interesting to take a look at the genes implicated in the transport and assimilation of carbon of *S. dimorphus* and *C. vulgaris*. More and more studies are being conducted on the genomics of microalgae. To product microalgal biofuel, genes implicated in the lipid production are being studied to get fast growing oil-rich microalgal strains (Bajhaiya *et al*., 2017; Xue *et al*., 2015; Guarnieri *et al*., 2018). Not much can be found on the genome of *S. dimorphus*. However, progress has been made on other microalgae of which *C. vulgaris*. Guarnieri *et al*. (2018) recently completed the genome sequencing of strain UTEX 395 of *C. vulgaris*, performed an assembly, created a gene set and measured the gene expression among several conditions. Molecular biology tools could be useful to detect any interaction between AM fungus and microalgae. The same kind of experiments as made in the context of this work, could be realized, microalgae could be sampled and RNA could be extracted (using a RNeasy Mini Kit (Qiagen) for example) in order to perform a PCR. In this view, primers of sugar transporters or genes implicated in nitrogen or phosphorus assimilation could be used. The genomic progress of microalgae should make it possible to detect proteins by mean of a PCR and better understand the exchanges of carbon between microalgae and mycorrhizae or detect any nitrogen and phosphorus depletion. For example, Xue *et al*. (2015) first discovered and characterized the Malic enzyme in microalga *Phaeodactylum tricornutum*, which is involved in pyruvate metabolism and carbon fixation. Moreover, the nitrogen assimilation inventory including genes for nitrate/nitrite transporters and reductases of *C. vulgaris* had also been made by Guarnieri *et al*. (2018).

VI. Conclusion

The main objective of this work was to identify an interaction between AM fungus and microalgae. For the first more applied objective, there was wanted to know how microalgae exudates influence spore germination and hyphal growth in a view to make biofertilizers/pesticides with microalgae exudates and spores. Two experiments studying spore germination, hyphal elongation and branching revealed that exudates of *S. dimorphus* and *C. vulgaris* cultivated in autotrophic (3N-BBM and MSR- medium) and heterotrophic (MSR+ 1g/L glucose medium) conditions have a stimulating effect on hyphal elongation and branching of *R. irregularis* MUCL 41833, *R. intraradices* MUCL 49410, *R. aggregatum* MUCL 49408 at a certain concentration up to a higher concentration, where no effect is observed anymore. The hypothesis was verified for exudates added in a solid MSR-and MSR+ (1g/L glucose) medium in Petri dishes on which spores were placed or encapsulated with spores in microbeads, placed in MSR- Petri dishes.

The findings also suggest that *C. vulgaris* produces different molecules or molecules in different quantities that influence hyphal branching when it is cultivated in autotrophic or heterotrophic conditions. On the other hand, similar quantities of *C. vulgaris* and *S. dimorphus* exudates cultivated in heterotrophic conditions induce the same hyphal elongation and branching. Although the three strains of mycorrhizae tested here (*R. irregularis* MUCL 41833, *R. intraradices* MUCL 49410, *R. aggregatum* MUCL 49408) are sensitive to microalgae exudates, they all appear to be at different concentrations. These results open doors to a new valorization filed in agriculture for microalgae exudates, waste from biomass production as biofuels or other.

The second more fundamental objective was to determine if AM fungal hyphae would help microalgae to survive in a sugar-free environment in the dark, by bringing them sugars. This was not confirmed. Three experiments of microalgae cultures in liquid and gelled MSR- hyphal compartments with and free of hyphae in the dark suggested that the microalgae grew better in the absence of hyphae. The main hypothesis is that hyphae deplete the medium of crucial mineral ions for the microalga. How the microalgae get their source of carbon however, remains ununderstood.

In a natural environment many more factors come into play and there would be the possibility of many more interactions. Just because it doesn't seem to have the expected interaction in a controlled closed environment like the one imposed in the context of this work doesn't mean there aren't any. This work did not allow the implementation of a protocol that shows that AM fungus feed algae but it is not excluded that this does not happen under other conditions. In addition, some interesting observations of microalgae that appear to follow hyphae and roots and be using the flow along roots to move around suggest the interaction of microalgae and the AM fungus-plant association is worth further investigation.

VII. References

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VIII. Appendices

Appendix 1: Germination process of an AM fungal spore

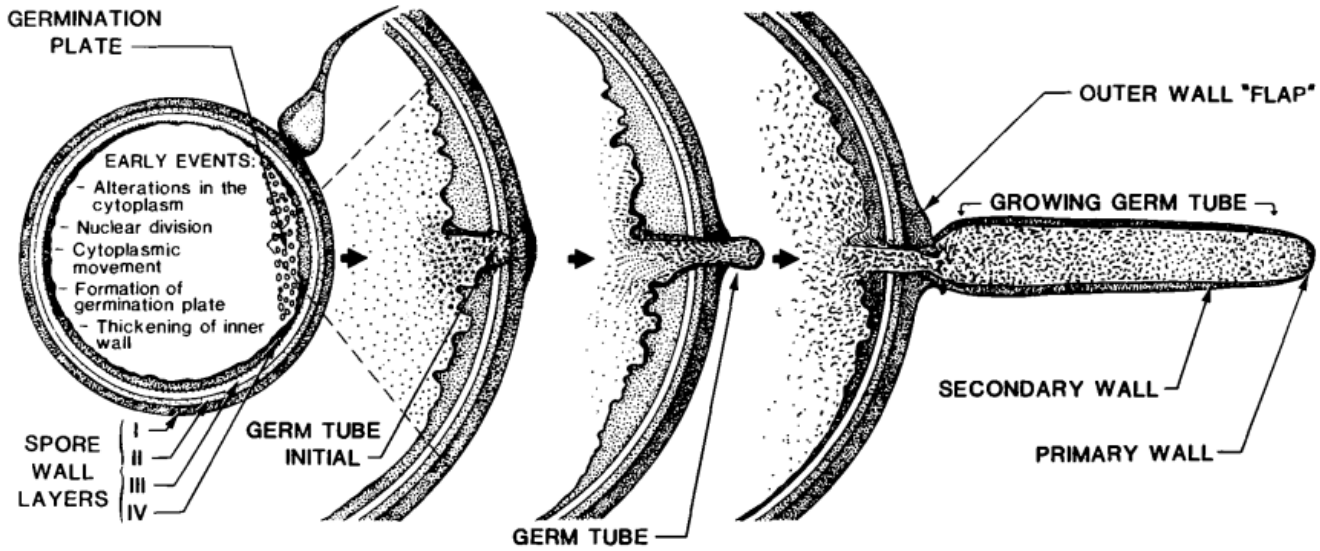

***Figure 52**: Diagramatic representation of the gross morphology of the germination process exhibited by Gigaspora margarita azygospores (Siqueira et al., 1985)*

Appendix 2: Preparation and composition of culture media

2.1 Milieu SRM (Strullu Romand Modifié) (Declerck *et al.*, 1996 modifié de Strullu et Romand, 1986)

International training on in vitro culture of Arbuscular Mycorrhizal Fungi (Declerck *et al.*, 2012).

Modified Strullu Romand (MSR) and minimum (M) medium preparation

Composition

In Table 1, the composition of the modified Strullu-Romand (MSR – Declerck *et al.*, 1998, modified from Strullu & Romand, 1986) medium and the minimum (M) medium (Bécard & Fortin, 1988) are given. These media are the most widely used (see Table 1 in Cranenbrouck *et al.*, 2005) for the *in vitro* culture of AM fungi. Although originally used for the growth of root-organs (e.g. Ri T-DNA transformed *Daucus carota* roots), it can be adapted to the HAM-P and AM-P *in vitro* culture systems to grow plants (e.g. *Medicago truncatula* and *Solanum tuberosum*) under autotrophic conditions by eliminating the sugar and vitamins.

Table 1. Mineral and vitamin composition of the modified Strullu-Romand (MSR) medium and minimal (M) medium (after Fortin *et al.*, 2002).

	MSR medium	M medium
$N(NO_3^-)$ (µM)	3800	3200
$N(NH_4^+)$ (µM)	180	-
P (µM)	30	30
K (µM)	1650	1735
Ca (µM)	1520	1200
Mg (µM)	3000	3000
S (µM)	3013	3000
Cl (µM)	870	870
Na (µM)	20	20
Fe (µM)	20	20
Mn (µM)	11	30
Zn (µM)	1	9
B (µM)	30	24
I (µM)	-	4.5

Mo (µM)	0.22	0.01
Cu (µM)	0.96	0.96
Ca Panthotenate (µM)	1.88	-
Biotin (µM)	0.004	-
Pyridoxine (µM)	4.38	0.49
Thiamine (µM)	2.96	0.3
Cyanocobalamine (µM)	0.29	-
Nicotinic acid (µM)	8.10	4
Glycine (mg/l)	-	3
Myo-inositol (mg/l)	-	50

The preparation of the MSR and M media requires the preparation of stock solutions (see Table 2).

Table 2. Concentration of stock solutions ($g.l^{-1}$) to prepare the MSR and M medium (after Fortin *et al.*, 2002).

		MSR medium	M medium
Macro-elements			
	KNO_3	7.6	8
	KCl	6.5	6.5
	KH_2PO_4	0.41	0.48
	$MgSO_4.7H_2O$	73.9	73.1
Calcium Nitrate	$Ca(NO_3)_2.4H_2O$	35.9	28.8
NaFeEDTA		1.6	1.6
Micro-elements			
	$MnSO_4.4H_2O$	2.45	6
	$ZnSO_4.7H_2O$	0.28	2.65
	H_3BO_3	1.85	1.5
	$CuSO_4.5H_2O$	0.22	0.130
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.034	-
	$Na_2MoO_4.2H_2O$	0.0024	0.002
	KI	-	0.75
Vitamins			
	Ca panthotenate	0.18	-
	Biotine	$0.18\ 10^{-3}$	-
	Pyridoxine	0.18	0.1
	Thiamine	0.2	0.1
	Cyanocobalamine	0.08	-
	Nicotinic acid	0.2	0.5
	Glycine	-	3
	Myo-inositol	-	50

Preparation of culture media

1. For 1000 ml of MSR or M medium mix together:
   - 10 ml of the macro-element stock solution.
   - 10 ml of the calcium nitrate stock solution.
   - 5 ml of the NaFeEDTA stock solution.

- 1 ml of the micro-element stock solution.
- 5 ml of vitamin stock solution to prepare MSR medium or 1 ml for M medium*.

2. Add 10 g of sucrose*.
3. After dissolution of sucrose, adjust the volume to 1000 ml.
4. Adjust the pH to 5.5 with NaOH or KOH and HCl.
5. Add 3 $g.l^{-1}$ Phytagel™, 3 $g.l^{-1}$ GelGro™, or 6 $g.l^{-1}$ agar.
6. Autoclave for 15 min. at 121°C under 1 bar pressure.
7. After autoclaving, the medium can be directly used but can also be stored for a few hours at 40-60°C.

Please notice that:

Gelling agents may contain diverse macro-, micro- and trace elements. It can therefore be critical to determine their presence and concentration (e.g. MSR medium solidified by Gel Gro™ contains actually 4.38 mM K and not 1.65 mM (see Table 1), therefore the difference (i.e. 2.73 mM K) is coming from the gelling agent).

Some gelling agents have been developed so that medium can be poured at low temperature (ca. 35°C).

Note that if Phytagel™ and GelGro™ can be dissolved using citrate buffer (0.01 M), agar and agar-based gelling agent cannot.

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*not needed for the preparation of medium for the HAM-P and AM-P *in vitro* culture systems

2.2: Chemical composition and preparation of 3N-BBM

Role	Compound	Concentration final solution (g*L-1)	Molecular Weight (g/mol)	Concentration final solution (M)
N source	**NaNO3**	**7,50E-01**	**84,99**	**8,82E-03**
N		1,24E-01	14,0067	8,82E-03
Mg source	**MgSO4**	**7,49E-02**	**246,47**	**3,04E-04**
Salinity	**NaCl**	**2,50E-02**	**58,44**	**4,28E-04**
pH buffer	**K2HPO4**	**7,51E-02**	**174,18**	**4,31E-04**
pH buffer	**KH2PO4**	**1,76E-01**	**136,0855**	**1,29E-03**
P		5,33E-02	30,973762	1,72E-03
K		1,06E-01	39,0983	2,71E-03
Ca source	**CaCl2**	**1,89E-02**	**110,98**	**1,70E-04**
Microelements	**ZnSO4 7H2O**	**8,83E-03**	**287,56**	**3,07E-05**
Microelements	**MnSO4 H2O**	**1,23E-03**	**169,02**	**7,28E-06**
Microelements	**MnCl2 4H2O**	**1,44E-03**	**197,91**	**7,28E-06**
Microelements	**(NH4)6Mo7O24 4H2O**	**6,09E-03**	**1235,86**	**4,93E-06**
Microelements	**Na2MoO4**	**1,19E-03**	**205,92**	**5,78E-06**
Microelements	**Na2MoO4.2H2O**	**1,92E-03**	**241,95**	**7,94E-06**
Microelements	**MoO3**	**7,10E-04**	**143,94**	**4,93E-06**
Microelements	**CuSO4 5H2O**	**1,57E-03**	**249,69**	**6,29E-06**
Microelements	**CoCl2 6H2O**	**4,00E-04**	**237,93**	**1,68E-06**
B source	**H3BO3**	**1,14E-02**	**61,833**	**1,85E-04**
complexing agent	**Na2EDTA 2H2O**	**6,37E-02**	**372,24**	**1,71E-04**
	KOH	**3,10E-02**	**56,11**	**5,53E-04**
Ferric solution	**FeSO4 . 7H2O**	**4,98E-03**	**278,01**	**1,79E-05**
	H2SO4 conc			1ml
	~~**B1**~~	~~1E-02~~		
~~Vitamines~~	~~**H**~~	~~2,5E-06~~		
	~~**B12**~~	~~1,5E-6~~		

After adding the different salts, the pH is adjusted to 7 and the medium is autoclaved before each use. To obtain an agar medium, 3g/L of phytagel is added.
After laboratory tests, the addition of vitamins (B1, H and B12), which are in principle necessary for certain strains of microalgae, is not necessary for the development of Scenedesmus strains.

Appendix 3: Preparation and composition of citrate buffer

International training on in vitro culture of Arbuscular Mycorrhizal Fungi (Declerck et al., 2018)

Citrate buffer

1. Citric acid 0,1 M (anhydrous) - Molecular Weight 192,13

2. Sodium citrate 0,1 M - Molecular Weight 294,10

Solution 1. Citric acid = 1,92 g/100 mL Solution 2. Sodium citrate = 2,94 g/100 mL

Take 1,8 mL of solution 1 and 8,2 mL of solution 2, adjust the volume to 100 mL with milli Q water, adjust to pH 6 with NaOH (1M)

The solution is sterilized using Acrodisc® filter with of 0,2 µm Suport® Membrane (PALL® Life science) before using.

To dissolve the gel:

1. Place a piece of gel in a petri dish and 10x volume of the buffer

   Ex: a gel block of 0,5 g, add 5 mL of buffer)

2. Cover the petri dishes with Parafilm® and keep in 25 °C of constant agitation for 15 minutes.

Appendix 4: Second trial of the impact of S. dimorphus and C. vulgaris MSR (+ and -) exudates on *R. irregularis* MUCL 41833 spores

Table 23: *Averages of germination rate and day, hyphal elongation and branching for 7 days of R. irregularis MUCL 41833 with varied concentrations of C. vulgaris and S. dimorphus MSR exudates: 2th trial for those who had an insufficient germination rate*

			R. irregularis MUCL 41833			
			GermRate	GermDay	DailyGrowth	DailyRam
CTRL		0%	0	0	0	0
C. vulgaris	light	0,25%	0,4	6,875	0,0506	0,212
		2,5%	0,222	7,5	0,0333	0,1563
	dark	0,25%	0,15	7	0,0126	0,125
		2,50%	0,316	9	0,0383	0,316
S. dimorphus	dark	2,50%	0,579	9,273	0,0151	0,147

Appendix 5: Pictures of experience 2 on gelled hyphal compartment

5.1: *C. vulgaris* of an E (microalgae and AM fungus in HC/ roots and AM fungus in RC) treatment of *R. aggregatum* MUCL 49408 on day 1.

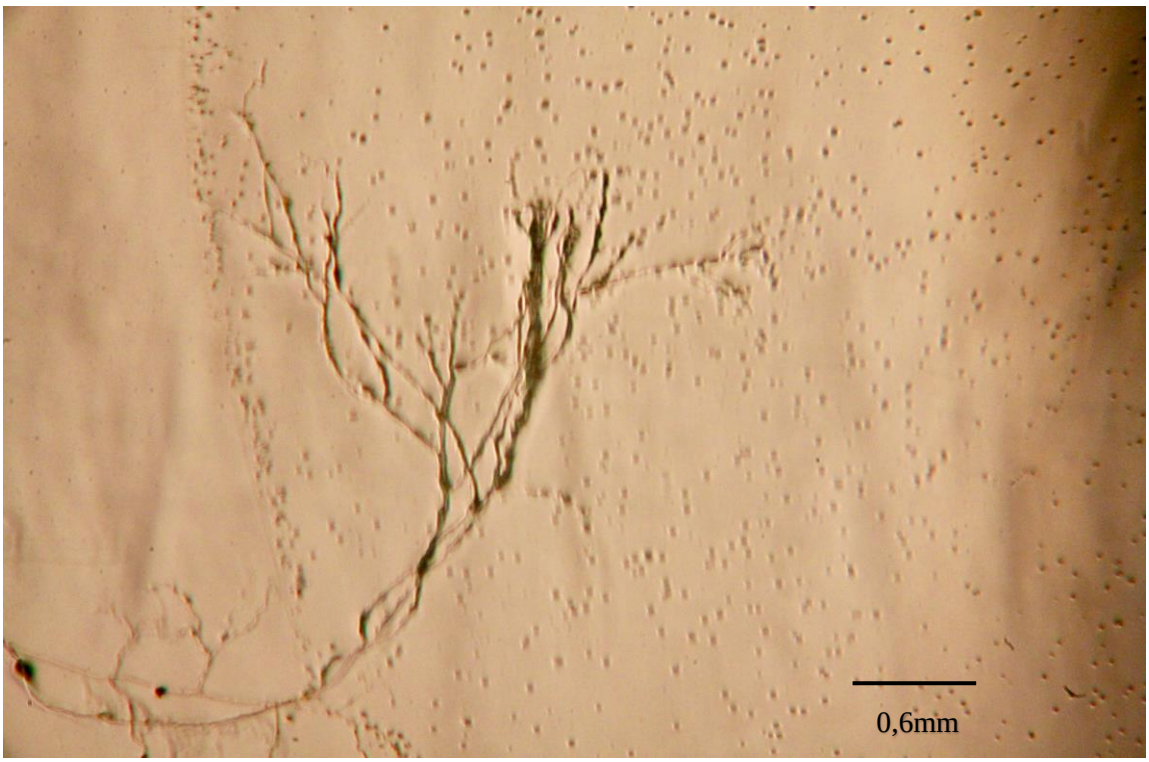

Figure 53*: C. vulgaris on R. aggregatum MUCL 49408 mycorrhized gelled hyphal MSR-compartment on day 1.*

5.2: *S. dimorphus* of a C (microalgae in HC/no roots no AM fungus in RC) treatment on day 1.

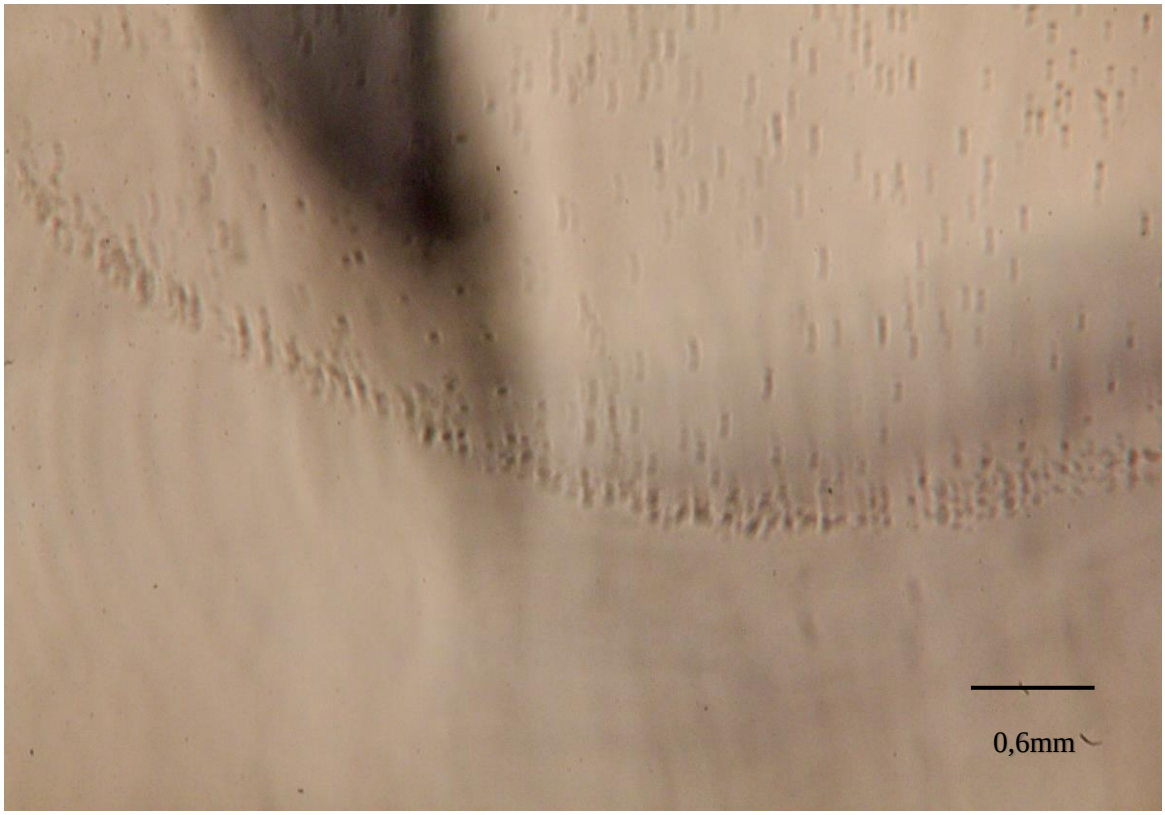

Figure 55: *S. dimorphus on non- mycorrhized gelled MSR- hyphal compartment on day 1.*

Evaluation *in vitro* de l'interaction entre les microalgues *C. vulgaris* et *S. dimorphus* et la symbiose mycorhizienne de champignons mycorhiziens à arbuscules du genre *Rhizophagus*

Présenté par Céline Van de Merckt

In the context of research for sustainable development the limitation of chemical inputs in agriculture and more extensive ways of producing food are in demand. Similarly, the production of biofuels and materials from renewable resources is highly required. Therefore, the demand of biomass of all kinds and in particular from microalgae is growing. These sub-products, the microalgae supernatants containing many compounds with diversified functions are looking for new valorization fields. The effects of these bio-sourced compounds on plants and mycorrhizal associations are relatively unexplored. However, some previous studies have open perspectives for their potential uses for stimulatory effects on the arbuscular mycorrhizal fungi. These obligate root symbionts improve hydric and mineral nutrition of plants and increase their resistance to abiotic and biotic stresses. A stimulation of the mycorrhizal association by microalgae exudates could help the fungi in reducing the use of agrochemical in agriculture production.

The focus of this work is set on the interaction between microalgae and AM fungi. In two first experiments, in line with a more applied approach, we aim to characterize the effects of molecules/components issued from microalgae on the germination of AM fungal spores and their hyphal growth. They revealed promising results in regard to the applied objective of the use of microalgae exudates and spores as substitute to agricultural biochemicals. Taken together, the findings suggest the microalgae exudates have stimulating effects on hyphal elongation and branching at a certain concentration up to a threshold concentration, where no effects are observed.

A third experiment tries to answer a more fundamental question; Does the AM fungus provide sugars to the microalga in a dark environment with no source of sugar? Although this hypothesis was not verified within the framework imposed by this work, interesting observations were made regarding the interaction between *C. vulgaris* and *S. dimorphus* and the AM fungus-plant symbiosis that opens up perspectives for further research.

Key words: Arbusculate mycorrhizal fungi, microalgae exudates, In vitro, C. vulgaris, S. dimorphus

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