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# Impact of elevated temperature on the association between *Crotalaria spectabilis* and different species of arbuscular mycorrhizal fungi

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# 2 Summary

Impact of elevated temperature on the association between *Crotalaria spectabilis* and different species of arbuscular mycorrhizal fungi:

As predicted by scientific models the world climate is changing and terrestrial temperatures are expected to increase by the end of the century. Arbuscular Mycorrhizal Fungi (AMF) are widespread soil microorganisms forming symbiotic associations with a majority of plant species, increasing their growth and resistance to biotic and abiotic stresses. Understanding the effect of increasing temperatures on these plant symbionts is thus of paramount importance.

Some studies have reported the effects of increasing temperatures on plants and AMF. A number of results concerned the root colonization by the fungus or the plant biomass. Concerning AMF sporulation, increasing as well as decreasing numbers of spores have been mentioned. However, no study was conducted under strict *in vitro* culture conditions, allowing to circumvent any unwanted parameters, and compared different AMF species.

Originally this master thesis aimed to study (1) the impact of the temperature on several mycorrhizal parameters and (2) the importance of the fungal intraspecific diversity in the mycorrhizal response to an increased temperature. Because of the COVID and a time restriction, the number of strains of AMF studied has been reduced making the studying of this second aspect difficult. Finally, this thesis has mainly concerned the impact of the temperature on the sporulation of AMF.

Two strains belonging to two different species of AMF (*Rhizophagus irregularis* and *Rhizophagus diaphanus*) have been inoculated to plants (*Crotalaria spectabilis*) under *in vitro* conditions. The plant-AMF associates were grown in growth chambers at two different temperatures and the spores produced were counted at regular intervals. Results of these counts have been analysed with a linear mixed model in R.

It can be seen from the best suited model that temperature has a significant effect on the spore production among the fungal species studied for the period and the experimental conditions considered. During the first four weeks the systems placed at 27 degrees Celsius had a higher sporulation that the systems placed at 24 degrees. But after the fourth week, the sporulation of the 24-degrees systems became higher, showing an interaction between the temperature treatment and the time. The temperature impact on the two AMF species was not significantly different.

Several ways have been proposed to improve the experiment and the experimental design for experiments similar to this one in the future. The effect of the temperature and that from the access to the water could be better discriminated. An analysis involving "S-shape" curves could also be better than an analysis involving the adjusting of a straight line to the data of each system. Finally, an automatic or semi-automatic counting of the spores should be envisaged seeing the effort required for the counting of one system when the sporulation is abundant. Programs already exist for the measuring of hyphae and roots for example.

# 3 Résumé

Impact d'une hausse de température sur l'association entre différentes espèces de Champignons Mycorrhiziens Arbusculaires (CMA) et *Crotalaria spectabilis* :

Selon les prédictions scientifiques, le climat change et les températures terrestres seront plus élevées à la fin de ce siècle. Les CMA sont des microorganismes du sol très répandus qui forment des associations symbiotiques avec la majorité des espèces de plantes et augmentant leur croissance et leur résistance à divers stress biotiques et abiotiques. Comprendre l'effet de l'augmentation des températures sur ces symbiontes est dès lors d'une grande importance.

Des études rapportent des effets de températures plus élevées sur les plantes et les CMA. De nombreux résultats concernent la colonisation racinaire par les champignons et la biomasse de la plante. En ce qui concerne la sporulation du champignon, des augmentations ainsi que des diminutions de la sporulation ont été rapportées. Cependant, il n'y a pas d'études conduites en conditions *in vitro* strictes sur des plantes entières, permettant ainsi de contrôler au maximum l'environnement, et comparant différentes espèces de CMA.

Ce mémoire avait originellement pour but d'étudier (1) l'impact de la hausse de température sur différents paramètres mycorrhiziens ainsi que (2) l'importance de la diversité intraspécifique du champignon dans la réponse mycorrhizienne à la température. Suite au COVID et à une restriction au niveau du temps, le nombre de souches de CMA a été réduit ce qui limite l'étude de ce second aspect. Finalement, le travail a principalement porté sur la sporulation et l'impact d'une température élevée sur celle-ci.

Deux souches appartenant à deux espèces différentes de CMA (*Rhizophagus irregularis* et *Rhizophagus diaphanus*) ont été inoculées à des plantes (*Crotalaria spectabilis*) en conditions *in vitro*. Les associations plante-CMA ainsi formés ont été placés dans des chambres de cultures à deux températures différentes et les spores produites ont été comptées à intervalles réguliers. Les résultats de ces comptes ont été analysés à l'aide d'un modèle linéaire mixte, dans R.

Il ressort des analyses que la température a un impact significatif sur la sporulation chez les deux souches étudiées, dans l'intervalle de temps et les conditions expérimentales considérés. Durant les quatre premières semaines, la sporulation des systèmes placés à 27°C était supérieure à celle des systèmes à 24°C. Après ce laps de temps, la sporulation des systèmes à 24°C est devenue supérieure, montrant l'existence d'une interaction entre le traitement de température et le temps. L'impact de la température sur les deux souches n'est, par contre, pas significativement différent.

Plusieurs pistes ont également été proposées pour améliorer le design expérimental et la méthode en cas d'expérience similaire future. Mieux séparer l'effet de la température de celui de l'accès à l'eau est une première piste. Une analyse impliquant une courbe en "S" serait également plus adaptée qu'une analyse basée sur l'ajustement d'une droite aux données de chaque système. Enfin un comptage automatique ou semi-automatique des spores devrait être envisagé vu l'effort que demande le comptage des spores chez une seule plante lorsque la sporulation est abondante. Des programmes existent déjà pour la mesure d'hyphes et de racines, par exemple.

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| | |
|---|---|
| AIC | Akaike Information Criterion |
| AM(s) | Arbuscular Mycorrhiza(s) |
| AMF | Arbuscular Mycorrhizal Fungus/Fungi |
| C | Carbon |
| CMA | Champignon Mycorrhizien Arbusculaire |
| GHG | GreenHouse Gases |
| GINCO | Glomeromycota *IN vitro* COllection |
| IPCC | Intergovernmental Panel on Climate Change |
| MSR | Modified Strullu-Romand (culture medium) |
| N | Nitrogen |
| NA | Non Available |
| P | Phosphorus |
| RCP | Representative Concentration Pathway (in IPCC reports) |
| Rd | *Rhizophagus diaphanus* |
| Ri | *Rhizophagus irregularis* |
| ROS | Reactive Oxygen Species |
| sqrt | Squared root |

# 7 Introduction and State of Art

## 7.1 Introduction

Great efforts are made to understand the effect of the climate change on the association between plants and Arbuscular Mycorrhizal Fungi (AMF). It is easily understood given the wide importance of this association around the world and through the plant kingdom. After a brief description of the arbuscular mycorrhiza and a short review of the research concerning the impacts of increasing temperatures on these fungi, a manipulative experiment and the results will be presented. It is about the impact of an increased temperature on the spore production of different strains of AMF grown *in vitro*. This study takes place in the framework of the DIVERCE project entitled: "*Does Intraspecific Variability modulate the impact of EnviRonmental Change on biodiversity and Ecosystem function?*" Among other objectives this project aims to study the impacts of the intraspecific diversity on the response of AMF communities to environmental change drivers (pollution by pesticides and temperature)[2].

## 7.2 Basic knowledge on arbuscular mycorrhizas

### 7.2.1 Definition, morphology and abundance of arbuscular mycorrhizas

Mycorrhizas may be defined as a non-pathogenic symbiotic relationship between a plant root and a fungus (Fitter *et al.* 1996). There are several types of mycorrhizas, as illustrated in Figure 1. An Arbuscular Mycorrhiza (AM) is a mycorrhiza where the fungus belongs to the *Glomeromycota* phylum (Schüßler *et al.* 2001). These fungi are called Arbuscular Mycorrhizal Fungi (AMF) because of the arbuscular structures they develop along the plasmic membrane of the cortex cells in the plant roots (Roth *et al.* 2017). AMF form a network of root-like structures. The network is called the mycelium and these structures the hyphae. A part of the network (the extraradical mycelium) extends outside of the roots and explores the soil to extract water and nutrients. It also produces the spores. Another part (the intraradical mycelium) colonizes the roots and bears the previously mentioned arbuscules and storage structures, the vesicles (for some genera) (see Figure 1).

AM is near ubiquitous in the terrestrial plant phylogeny. A study shows for example that most of the species and families of terrestrial plants (80 and 92% respectively) for which the presence/absence of mycorrhizas have been recorded are mycorrhizal (Wang *et al.* 2006). Most of these families form AMs (Wang *et al.* 2006). Some researchers give an estimation of two thirds of terrestrial plant species being involved in AM (Fitter *et al.* 1996). These numbers, if they must be considered cautiously[3], give an idea of the abundance of mycorrhizas, especially AM, in the plant kingdom.

[2] More details on this project may be found on this official website of the European Union: https://euraxess.ec.europa.eu/jobs/325237

[3] See Fitter *et al.* 1996 for a criticism of the advanced two thirds.

Geographically, the *Glomeromycota* phylum is distributed worldwide. It can be found on the seven continents, in 10 of the 11 biogeographic realms and in the 14 biomes[4] (Stürmer *et al.* 2018). The four Orders in *Glomeromycota* (i.e.: *Diversisporales*, *Glomerales*, *Paraglomerales* and *Archaeosporales*) are also found in most of the continents (6 or 7, depending on the Order), in most of biogeographic realms (from 9 to 10 on 11, depending on the Order) and in most of the biomes (from 10 to 14 on 14, depending on the Order) (Stürmer *et al.* 2018). At the species level, Stürmer *et al.* (2018) have found 43% of the AMF species (on 222 species studied) being cosmopolitan (found in 4-7 continents) and 26% of them being endemic to one continent.

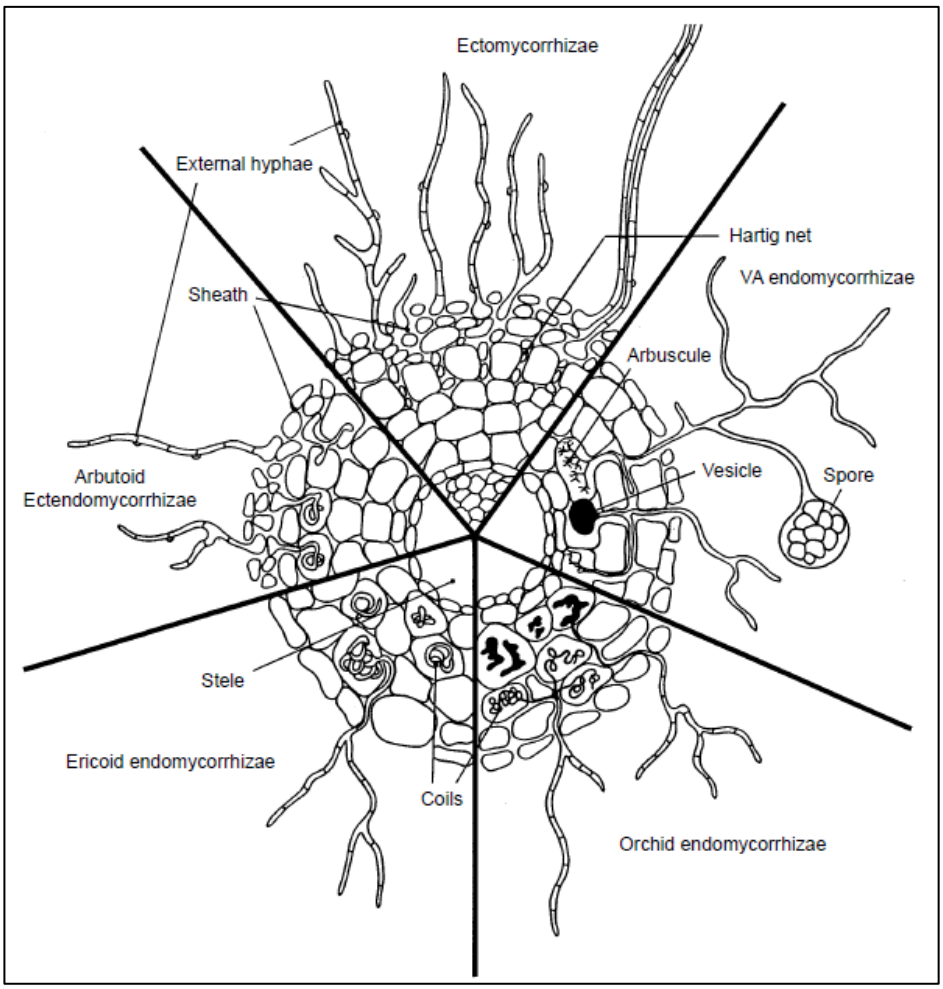


*Figure 1 : Illustration of the different types of mycorrhizas. Today, the "VA endomycorrhizae", Vesicular-Arbuscular endomycorrhizae, is called the arbuscular mycorrhiza (AM). We can see the AM extraradical and the intraradical mycelium. Particular AM structures like a spore, a vesicle and an arbuscule are also visible. (Selosse et al. 1998)*

### 7.2.2 Carbon transfers

[4] Biogeographic realms as they have been defined by Holt *et al.* in 2013 and biomes by Olson *et al.* in 2001 (Stürmer et al. 2018)

Different transfers of nutrients take place in the AM. Indeed, the plant provides carbon (C) to the AMF while the fungus provides various nutrients to the plant (for example phosphorus (P), nitrogen (N), etc.) (Bucher *et al.* 2007, Smith *et al.* 2011b). Concerning C transfers, the AMF is a heterotroph and depends on the C provided by the plant as hexoses (Bago *et al.* 2000) and possibly as lipids (Rich *et al.* 2017, Keymer *et al.* 2018). Colonized plants generally allocate five to ten per cent more C (up to 20 per cent) to their root system in comparison with their non-colonized counterparts (Bago *et al.* 2000, Fitter *et al.* 2011). Consequently, AMF strengthen the sink effect of the root system.

### 7.2.3 Phosphorus transfers

Phosphorus (P) transfers are important for plants involved in AM especially because this element is involved in some highly important biological molecules (DNA, ATP, some lipidic compounds of the membranes, etc.) and because it has a limited bioavailability. Indeed, P is present as a negatively charged ion in the soil which forms poorly soluble complexes with some cations (iron, aluminium or calcium, according to the pH) with a poor mobility of P as a consequence (Smith *et al.* 2011a). Additionally, the cytoplasm of root cells is negatively charged and the P concentration in these cells is greater than the P concentration in the soil. As a result, the plants have to uptake P actively (Smith *et al.* 2011a). With its dense mycelium of long and fine hyphae the AMF can extend outside of the P depletion zone surrounding plant roots. AMF can take up inorganic P in un-depleted zones and transfer it to the plant (Smith *et al.* 2011a, see Figure 2). They can also possibly increase the amount of soil available P by the release of acid phosphatases and the associated release of P from organic compounds (Sato *et al.* 2015). In some cases, the transfer of P by AMF leads to a higher plant biomass but sometimes the response of the plant is null or negative. However, some studies show that even when such null or negative answers happen the P transfer by the AMF may account for a major contribution to the total P uptake of the plant (Smith *et al.* 2011b).

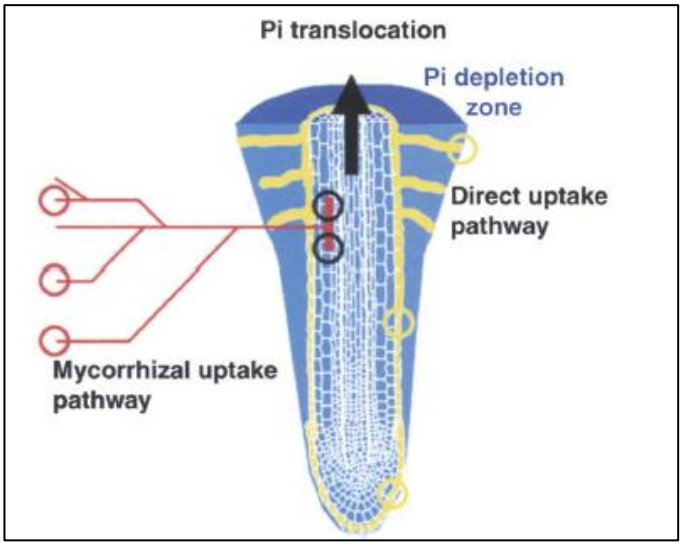


*Figure 2 : Illustration of the different pathways for P uptake in AM plants. Plants uptake P directly by their root hairs (direct pathway) or through AMF uptake (mycorrhizal pathway). AMF mycelium can extend outside of the depleted zone close to the roots. Colour circles represent P transporters on hyphae, on root epidermis or on the interface between the hyphae and the cortical cells. (Bucher et al. 2007)*

### 7.2.4 Nitrogen transfers

Nitrogen (N) transfers also happen from the AMF to the roots although they have been historically neglected despite some evidence of such fluxes. Nitrate is supposed to be the principal source of N for plants in the ecosystems where AMF are the most abundant. And the higher nitrate mobility made the intervention of AMF for N uptake in favour of the plants unnecessary (Hodge *et al.* 2015). However recent studies show that AMF are capable of transferring N from inorganic and organic sources to their host (Hodge *et al.* 2015, Schroeder-Moreno *et al.* 2012). Quantification of N fluxes to the plant is difficult and currently results are very variable from one experiment to another (Hodge *et al.* 2015, Smith *et al.* 2011b).

### 7.2.5 Other nutrients transfers

AMF can also influence the micronutrients content of their host, for example the zinc, the copper and the manganese content. The nature of the influence (increase or decrease of the plant content) depends on environmental factors like the mineral content of the soil (Liu *et al.* 2000).

### 7.2.6 AMF and their ecosystems

Beyond effects on their hosts AMF also have impacts on their ecosystems. For instance, they influence the soil where they develop. They impact the soil biochemistry by their secretions and their metabolism, by the decomposition of their hyphae and by their action on the plant metabolism (Rillig *et al.* 2006). They also influence the soil structure by the presence of their mycelium, by the pressure they exert on the soil particles and by their secretions (Rillig *et al.* 2006). Moreover, the AMF community and its diversity may also have an impact on the plant community structure (relative abundance and diversity) and its productivity (Hartnett *et al.* 1998, Zhang *et al.* 2016, van der Heijden *et al.* 1998).

## 7.3 Climate change predictions

Because of the large abundance of AMF and the influence they have on plants and ecosystems it is important to study the effects of future climate change on the AM. In this work, we will use the data and predictions reported by the IPCC as the reference for the future climate (IPCC 2014).

According to the predictions of IPCC climatic models, mean global surface temperature is going to increase by 0.3 to 4.8 degrees Celsius on the period between 1986-2005 and 2081-2100, as it is reported in the Table 1 (IPCC 2014). The increased temperature is expressed as a range of temperatures because the increase depends on the scenario of greenhouse gases (GHG) emissions entered in the models. The warming on the lands is expected to be higher

than that on the oceanic areas and consequently higher on the lands than the means given in the Table 1 (IPCC 2014).

*Table 1 :* Changing in degrees Celsius of the mean global surface temperature between the period 1986-2005 and the period 2081-2100. RCP (Representative Concentration Pathway) are the possible GHG emission profiles which determine the future climatic scenarios. RCP 2.6 is a low emission profile, RCP 4.5 and 6.0 are intermediate emission profiles and RCP 8.5 is a high emission profile leading to the highest climatic change. The range of confidence comprises uncertainties in the models and in the scenarios (adapted from IPCC 2014).

| **Climatic scenario** | **Mean rising (°C)** | **Range of the rising (°C)** |
|---|---|---|
| RCP 2.6 | 1 | 0,3 -1,7 |
| RCP 4.5 | 1,8 | 1,1-2,6 |
| RCP 6.0 | 2,2 | 1,4-3,1 |
| RCP 8.5 | 3,7 | 2,6-4,8 |

## 7.4 Impacts of temperature on the arbuscular mycorrhizas

### 7.4.1 Overview of the diversity of approaches

This section aims to show the huge diversity of methods and biological materials used in the literature on AM and temperature[5]. This section constitutes then a review of the approaches used in the literature, while the next section reviews the results and discussions.

#### *7.4.1.1 Overview of the field*

There are many papers concerning the impacts of temperature on the AM symbiosis in the literature. Looking at the different publications we can distinguish two waves of studies on the subject.

The first one occurred during the three last decades of the twentieth century where many papers were published (ex. Grey W.E. 1991, Gunasekaran P. *et al.* 1987, Monz *et al.* 1994, Schenck *et al.* 1975, Schenck *et al.* 1982, Smith *et al.* 1986). They mostly present laboratory studies and do not concern climate change. The scientists aimed for instance to identify optimal temperature for the culture of AMF, to obtain a maximum of spores and colonized roots for plant inoculations (Schenck *et al.* 1982).

The second wave encompasses the two last decades and concerned mostly climate change (but also see Monz *et al*. already published in 1994 for example). During this period the domain has flourished with many new studies. They are quite different from the first ones:

[5] By the terms “temperature” and “temperature treatment”, we mean in this document an increased temperature and a treatment with an increased temperature.

several studies involved different abiotic factors (not only the temperature) and many of the studies of this period were done in the field.

#### *7.4.1.2 The experimental context*

Some studies were done in chambers (ex. Hawkes *et al.* 2008), others in the field (ex. Wilson *et al.* 2016) and some contained both chambers and field experiments (ex. Bunn *et al.* 2009).

Among the field experiments we can find several ecosystems and climatic conditions studied. There are studies in fields in warm-temperate continental climate (Tian *et al.* 2019), in grasslands under Mediterranean climate (Wilson *et al.* 2016, Rillig *et al.* 2002), in temperate meadows (Zhang *et al.* 2016, Mei *et al.* 2019a), in semi-arid meadows (Mei *et al.* 2019b) and in grasslands under temperate oceanic climate (Heinemeyer *et al.* 2003, Staddon *et al.* 2003), for example.

#### *7.4.1.3 The biological material*

Scientists have used many different species of plants. Some have used transformed carrots roots in *in vitro* systems (ex. Costa *et al.* 2013), others have used agronomic species (ex. Tian *et al.* 2019) and others have used natural species (ex. Rasmussen *et al.* 2020). Some have used one plant species (ex. Pischl *et al.* 2017) while other scientists have studied the whole plant community (ex. Staddon *et al.* 2003).

There is also a diversity of AMF species used. Some researchers utilized one or a few species inoculated (ex. Martin *et al.* 2003) and others worked with the whole AMF community (ex. Birgander *et al.* 2017).

#### *7.4.1.4 Simulating the future climate*

As we mentioned in the previous section, climatic models can only give us a range of temperature as predictions for the future climate because of the uncertainties and the different parameters entered. Consequently, the scientists who have worked on rising temperature and AM have used a wide variety of temperatures to simulate climate change. Most of the studies involved a treatment with a temperature elevation from one to five degrees but some scientists have used a higher one (Rasmussen *et al.* 2020, Heinemeyer *et al.* 2004). In contrast, most of the studies of the last century ("first wave") implied several temperature treatments with a wide range of temperatures like Schenck *et al.* (1982) who worked in their article at 18, 24, 30 and 36 degrees. Many studies in the field were realized with electrical heaters above the vegetation to obtain the warming treatment (ex. Tian *et al.* 2019 or Wilson *et al.* 2016). Such systems imply both a warmed soil and a warmed atmosphere around the heaters. These systems imply that the AMF and the plants are both under the warming treatment. In the laboratory experiments the ways the systems were warmed are more various. Heinemeyer *et al.* (2004) for instance have realized an experiment where the plant and the AMF were both warmed and a second one where only the AMF extra-radical

mycelium was warmed via a heated hyphal compartment. They designed these experiments to distinguish the direct effects of the temperature on the extra-radical mycelium from the effects mediated by the warming of the host plant. Other experiments imply systems where only the soil temperature is manipulated and where the AMF and the plant root system are warmed but not the plant shoot (ex: Gavito *et al.* 2003 or Grey *et al.* 1991).

#### *7.4.1.5 Experiment duration*

Most of the studies on AM and temperature last from several weeks to a growing season. Some studies are quite longer and last several years. Most of them are field studies (Birgander *et al.* 2017, Mei *et al.* 2019a, Rillig *et al.* 2002, Staddon *et al.* 2003, Tian *et al.* 2019, Wilson *et al.* 2016 but also see Monz *et al.* 1994 for a long laboratory study). In their review of studies on mycorrhizal responses to global change Mohan *et al.* also highlight the great majority of short-term studies (Mohan *et al.* 2014).

#### *7.4.1.6 Variables measured*

Researchers working on AM and temperature have measured many different variables. Concerning the plants, they have for instance measured the shoot and the root biomass, the root-shoot ratio, the plant content and/or concentration in N, P and C, the yield, the plant height, the number of flowers and/or the number of fruits. Concerning the fungi scientists have for example measured the hyphal length density, the germination rate of the spores, the respiration rate by the extraradical mycelium and/or the percentage of root length colonization (by hyphae, by arbuscules and/or by vesicles). Some studies have been done at the scale of the community. Such studies have variables such as the diversity and the relative abundance in the plant community and/or the composition of the AMF community. The number of variables and the choice of them vary widely among studies depending on the aspects of the AM the scientists were interested in.

### 7.4.2 Main results in the literature

As said earlier, after the description of the wide variety of approaches and materials used, we are going to expose the main results of the literature on the temperature impacts on the AM. The root colonization by the AMF and the plant biomass are probably the two variables studied the most frequently. For this reason, we are first going to have a look at the results concerning these two characteristics. After them, results of some other variables often encountered will also be analysed.

#### *7.4.2.1 Root colonization by AMF*

We can find publications where higher temperature has positive (ex: Gavito *et al.* 2003, Kytöviita *et al.* 2007, Schenck *et al.* 1982) as well as negative (Tian *et al.* 2019, Wilson *et al.*

2016) or no effects (Schroeder-Moreno *et al.* 2012, Pischl *et al.* 2017, Hawkes *et al.* 2008) on the root colonization by AMF. Analysing the literature, we have found more often cases where the temperature has positive or no effect on the root colonization. However, this trend must be taken with great care. Searching for a significant trend in the literature would require statistical tools, an approach we cannot afford in this state-of-art[6].

By looking at the variability within papers, we can see that the effect of temperature on AMF root colonization is dependent on the AMF and the plant species involved. For instance, Martin *et al.* (2004) have found in the same experiment a positive effect for the colonization of pepper (*Capsicum annuum*) by one AMF species (*Glomus sp.*) and a negative effect for another AMF species (*Rhizophagus irregularis*). In another experiment the temperature had a positive effect on the colonization of one plant species (*Leymus chinensis*) and no effect on the colonization of another plant species (*Puccinellia tenuiflora*) by the AMF community (Mei *et al.* 2019a). Monz *et al.* (1994) also observed different effects of temperature on the colonization of different plant species (*Pascopyrum smithii* and *Bouteloua gracilis*).

In addition, we have to report a temporal variability in the results. For example, Rillig *et al.* (2002) have observed a significantly increased root colonization with higher temperature one year, but they had no significant increase the previous year. Heinemeyer *et al.* (2003) made three experiments in one of their publications: two short experiments of one season and a bigger one of one year. They observed no impact of soil temperature on colonization in the two first experiments but observed a negative effect in the one-year experiment. Even if we look only at long-term experiments to try to avoid temporal variability, we can find both positive (Staddon *et al.* 2003, seven years) and negative effects (Tian *et al.* 2019, Wilson *et al.* 2016, two years each).

Many researchers do not discriminate direct from indirect effects of temperature on AM. Temperature can affect the symbionts (plant and AMF) directly, by affecting their metabolism for example, but it may also impact the symbionts by affecting other environmental parameters like the availability of soil water and nutrients (Wilson *et al.* 2016). The Figure 3, from Wilson *et al.* (2016), is an illustration of Structural Equation Modelling showing such direct and indirect effects of temperature on AMF colonization. Let us have a look at one indirect effect, the humidity level, for example. Some searchers have taken it into account (Tian *et al.* 2019) while others have not (Mei *et al.* 2019a) making the comparison of net effects of temperature on root colonization between different studies difficult. However, in their study, Wilson *et al.* (2016) have obtained positive and negative indirect effects cancelling each other out. In their article the direct effects of temperature are the most important. Moreover, some authors also include other global change drivers like N inputs, a higher $CO_2$ concentration, etc. in a combined "future climate treatment" (ex. Zavalloni et al. 2012) also complicating the comparison between studies.

The authors of two papers have observed for several AMF species a parabolic evolution (with a maximum value) of the root colonization rate with the temperature (Schenck *et al.* 1982, Gavito *et al.* 2005). For some species they obtained a linear rising curve in the temperature

[6] Kivlin *et al.* have made such a metanalysis on the effects of different climate change drivers on AM-plant biomass. They have searched for statistically significant trends among the wide variety of results. However, they did this difficult exercise only for the results concerning the plant biomass, studied later in this work (Kivlin *et al.* 2013).

range they utilized, but for these species a maximum may not have been attained within the temperature range they studied. Firstly, these authors showed a negative effect of low temperatures[7] on AMF colonization. Secondly, they showed the presence of a species-specific optimal temperature for root colonization. Such a pattern may account for a part of the variability in colonization results in the literature. It may for example explain the different effect found for different species in the same experiment reported above. For example, if the heating treatment brings one species closer to its optimal temperature range and the other species beyond its optimal range, we will respectively observe a positive effect and a negative one.

We can also question whether the increase in temperature will have the same effect on AMF colonization under different climates. The two low-Arctic plants (*Potentilla crantzii* and *Ranunculus acris*) studied by Kytöviita *et al.* (2007) have shown a positive response of root colonization to a higher temperature. However, it is the only study we have found in cold regions. Studies in warm regions would also be interesting to test what would be the temperature effect. Currently it is difficult to understand the answer of AMF in cold and warm countries because of a lack of studies. Mohan *et al.* (2014) in their review on mycorrhizal fungi and global change have also signalled a bias in the countries studied toward Europe and North America and a lack of studies in tropical and inter-tropical regions. We can add to their observation that many recent publications also come from Asian countries (ex: Tian *et al.* 2019, Mei *et al.* 2019b). Further studies in climatic regions with a lack of data would be interesting to understand the local effects.

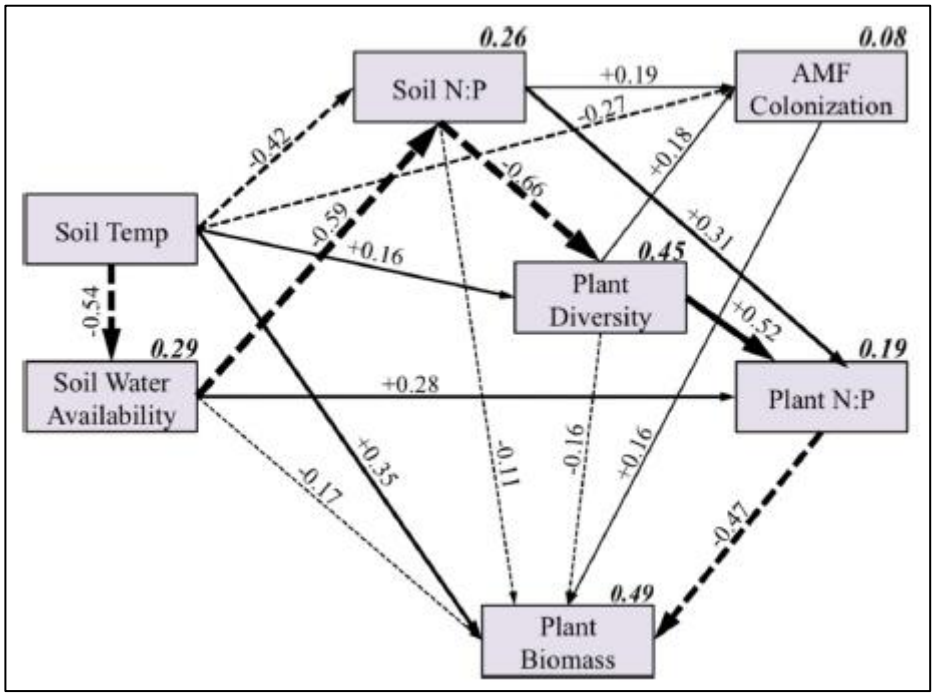


*Figure 3 : Structural Equation Modelling showing direct and indirect effects of temperature on root colonization by AMF and on plant biomass. The boxes of the figure are the variables of the model. The arrows have different width in function of the importance of the effect (solid arrows for positive effects and dashed for negative ones). Only the significant paths at p <*

[7] Law but positive temperatures. We have a chilling stress for the AMF, not a freezing stress.

*10% are shown. The numbers above the grey boxes represent the explained variance of each variable (R2). (Wilson et al. 2016)*

#### *7.4.2.2 Plant biomass*

One more time the variety of approaches makes the comparison of the results difficult. Some of the scientists have only measured the root biomass, the shoot biomass, the total biomass or a mix of them. Besides as for the root colonization the results are highly variable. Taking shoot biomass as an example we can find papers where the temperature is reported to increase it (Schroeder-Moreno *et al.* 2012, Rasmussen *et al.* 2020), to decrease it (Mei *et al.* 2019a) or to have no effect (Hawkes *et al.* 2008). As for the root colonization several papers suggested that the effects of temperature on AM plant biomass is different according to the plant species and the AMF species involved (Heinemeyer *et al.* 2004, Wilson *et al.* 2016, Martin *et al.* 2004).

Many interactions have been found with other factors making integrative predictions of the plant biomass response to elevation of temperature difficult. We can cite as examples an interaction between the presence of AMF, the nitrogen input and the temperature treatment (Zhang *et al.* 2016) or an interaction between the temperature treatment, the presence of AMF and the drought treatment (Pischl *et al.* 2017).

In a meta-analysis of the impacts of global change drivers on mycorrhizas, Kivlin *et al.* (2013) have obtained a majority of reported positive impacts of temperature on plant biomass in studies on AM. They also report that the AMF has no significant effect on plant biomass in these studies and that there is no interaction between temperature and the AMF presence for this variable.

#### *7.4.2.3 Phosphorus transfers*

Looking at results concerning P concentration[8], mainly foliar concentration, we mostly obtain increased or unchanged P concentration with temperature (Gavito *et al.* 2003, Mei *et al.* 2019a, Kytöviita *et al.* 2007, Smith *et al.* 1986). One study reported a significant interaction between the AMF treatment and the temperature and reported a decrease in foliar P concentration with temperature if the pepper plants are mycorrhizal (Martin *et al.* 2003). Another study employing a treatment with combined increased $CO_2$ and increased temperature showed no global impact of the combined drivers on the leaf P concentration among several AM plants (Zavalloni *et al.* 2012).

#### *7.4.2.4 Nitrogen transfers*

Results concerning the effects of temperature on the N flows in AM are scarce in comparison with the results on P flows. One study shows no effect of temperature on N concentration of plant leaves but a significant interaction between the presence of AMF and the warming

[8] Not the absolute P content, to avoid bias due to a change of the plant biomass from one experiment to another.

treatment. The temperature reduces slightly a negative impact of the AMF treatment on N concentration in one of the two plant species studied (*Leymus chinensis*) (Mei *et al.* 2019a). Another study showed a negative effect of temperature on shoot N concentration for two AM plant species (*Potentilla crantzii* and *Ranunculus acris*) (Kytöviita *et al.* 2007). One more study showed a negative effect of combined elevated temperature and $CO_2$ on the foliar N concentration and no effect of this treatment on the root N concentration of several AM species (Zavalloni *et al.* 2012).

#### *7.4.2.5 Sporulation and germination*

Several studies, mainly conducted in growth chambers, concerned the sporulation and the germination of AMF spores. Some of these papers report an increase in the soil spore density or in the number of spores produced with higher temperatures (Mei *et al.* 2019a, Wahl *et al.* 2016, Gavito *et al.* 2005). Other papers reported a negative effect of elevated temperatures on both sporulation and spore diameter (Zhang *et al.* 2016, Costa *et al.* 2013). The presence of a species-specific optimal temperature has been identified for the sporulation of different AMF species (*Glomus claroideum*, *G. mosseae*, *G. clarum*, *Gigaspora gregaria* and *Acaulospora laevis*) as for the root colonization (Schenck *et al.* 1982).

Concerning the germination rate of spores two publications show that some AMF species (*Gigaspora coralloidea*, *G. heterogama*) have a linear curve of germination rate with temperature on the tested temperature range and other species (*Glomus mosseae, G. fasciculatum*) have a parabolic curve with an optimal temperature (Schenck *et al.* 1975, Gunasekaran *et al.* 1987). Both cases suggested a negative effect of low temperature and a beneficial effect of higher temperature, at least to a certain temperature.

#### *7.4.2.6 Extraradical mycelium abundance*

Among the articles, most of the studies on the impact of temperature on the extraradical mycelium abundance showed a positive impact of the temperature treatment (Bunn *et al.* 2009, Gavito *et al.* 2003, Gavito *et al.* 2005, Mei *et al.* 2019a, Zhang *et al.* 2016). One paper reported a negative impact of temperature on the extraradical mycelium abundance (Staddon *et al.* 2003). Another paper also showed that the effect of temperature depends on the plant species involved in the AM. The authors found an increased extraradical mycelium for one plant species (*Plantago lanceolata*) but no impact for another (*Holcus lanatus*) in an experiment where both the plant and the AMF (*Glomus mosseae*) were under elevated temperature (Heinemeyer *et al.* 2004). In another experiment with *P. lanceolata* where only the soil of a hyphal compartment was heated, they found a positive effect of temperature on extraradical mycelium (*Glomus mosseae*, *G. hoi* and *Acaulospora sp*.) decreasing with time and not being significant anymore at the last harvesting showing a possible time effect (Heinemeyer *et al.* 2004).

#### *7.4.2.7 Arbuscules and vesicles abundance*

A few publications contained results about the impact of temperature on the abundance of vesicles and arbuscules. Concerning the arbuscules the results varied from one publication to another. Hawkes *et al.* (2008) report no temperature effect on arbuscules (*Rhizophagus irregularis* and *Glomus mosseae*) while Kytöviita *et al.* (2007) report an increased arbuscular abundance (*Glomus claroideum*) in the two plant species they studied (*Potentilla crantzii* and *Ranunculus acris*). One other publication showed an increased arbuscular abundance for an AMF species (*Glomus sp*.) and a decreased abundance for a second AMF species (*Rhizophagus irregularis*) (Martin *et al.* 2003).

Concerning the vesicular abundance one study showed a negative effect of temperature (Hawkes *et al.* 2008) and another showed a negative or no effect according to the AMF species involved (Martin *et al.* 2003). Heinemeyer *et al.* (2004) observed a positive impact of temperature on vesicular and arbuscular of one plant species studied (*Plantago lanceolata*) but no impact for the second plant species (*Holcus lanatus*) when both plant and AMF had the temperature treatment. In the other experiment where only the extraradical mycelium was under an elevated temperature the researchers have observed no change in the vesicular and the arbuscular abundance suggesting a plant impact on the morphological response of the AMF (*Glomus mosseae*, *G. hoi* and *Acaulospora sp*.) to temperature.

#### *7.4.2.8 Acquired heat tolerance*

In 1975, a publication on the effects of temperature on germination of AMF spores has suggested that AMF strains may be adapted to the temperatures of their original natural environment (Schenck *et al.* 1975). Using spores from Florida owing to two different species (*Gigaspora coralloidea* and *G. heterogama*) and spores from a third species (*Glomus mosseae*) coming from the state of Washington they showed that the spores from Florida germinated better at 34 degrees and the ones of the Washington state germinated better at 20 degrees. The authors suggested an adaptation of the strains to the temperature of the environment they came from.

Since this publication other searchers have studied if such a thermal adaptation exists. Using an AMF community from a thermal soil from Yellowstone National Park and another from a non-thermal soil of this park, Bunn *et al.* (2009) have not found any difference between the two communities by measuring different plants and AMF characteristics, in a temperature treatment experiment. Their results do not support an acquired heat tolerance at the scale of the AMF community. On the other hand, using an inoculation mixture of two AMF species (*Glomus macrocarpum* and *Glomus sp.)* from the Montana (USA) and a mixture of three AMF species (*Glomus hoi, G. geosporum* and *G. fasciculatum*) from Syria, Grey (1991) has shown a greater tolerance of the fungi from Montana to cool soils and a greater tolerance of the Syrian AMF to warm soils regarding root colonization in *Hordeum vulgare*. It supports acquired heat tolerance. But, in their study, Gavito *et al.* (2005) used two strains of AMF species coming from Canada (*Rhizophagus irregularis* and *Glomus cerebriforme*) and one strain of another species (*Glomus proliferum*) coming from Guadeloupe and they obtained no results in favour of an acquired heat tolerance.

In the cited publications the authors compared strains of different species or compared different AMF communities. Maybe the comparison of strains of the same species coming

from different climatic conditions would be more adequate to test a possible acquired heat tolerance without confusing the species effect and the origin effect. This small comment brings us to the mention of another lack in the current knowledge on the effect of temperature on AM: we have not found studies on the impact of the intraspecific variability on the effect of warming on AM in the literature. Maybe the intraspecific variability could explain a part of the variability observed in the results for the different variables presented.

#### *7.4.2.9 Summary of the results*

There is often a high diversity in the results concerning the temperature effect on the parameters of the AM-plants and the AMF. Having a look at the diversity of methods and the diversity of material used this variety in the results is not so surprising. Below, the results per variable are successively summarized.

- Many studies have reported impacts of temperature on AMF root colonization either positive, negative or neutral. Positive and neutral effects are the most common, but the results depend on the plants and the AMF species studied. Some results showed a species-specific parabolic (with an optimum) evolution of root colonization rate with temperature. The literature is geographically biased and experiments in cold and warm (tropical, sub-tropical) environments would be interesting for a global comprehension of the impact of temperature.
- Results concerning the plant biomass also showed a great diversity of responses. The plant biomass response to temperature depends on the plant species and the AMF involved. A meta-analysis shows no significant interaction between the AMF presence/ absence and the general positive response of plant biomass to the temperature treatment.
- Concerning P, most of the results supported a positive or no effect of temperature on the P concentration of AM-plants.
- Most of the results supported a negative or no effect of temperature on N concentration in AM-plants but results are scarce and extra studies are needed to check if we may be confident in the observed trend.
- Both positive and negative effects of elevated temperature have been found for the production of spores. Several papers showed a positive linear or a parabolic evolution for different AMF species (as for colonization) for both spore production and germination rate.
- The majority of results concerning extraradical mycelium abundance showed a positive effect of temperature although it may vary with the plant species involved.
- It is difficult to identify a pattern in the results concerning the abundance of arbuscules or vesicles because of the variety of results obtained and the scarcity of the results available. The AMF response seemed dependent on the species involved in the AM and one more time more work is needed to see if a response is more frequent than another.

- Different scientists have tried to test if an acquired heat tolerance by AMF strains exists. There are few results and some are in favour of such a tolerance and others do not support this hypothesis. Maybe the comparison of different strains of the same species but coming from different climatic areas could help to investigate this interesting question. The lack of consideration to AMF intra-specific diversity among all the papers reported must also be highlighted.

# 8 Material and method

## 8.1 Biological material

The plant species used in this experiment is *Crotalaria spectabilis*, an annual dicot coming from Asian tropical countries.

The AMF belong to two different species: *Rhizophagus irregularis* (previously called *Glomus intraradices*) and *Rhizophagus diaphanus*. The strain of *R.irregularis* is registered in the collection of the lab (GINCO, *Glomeromycota IN vitro* COllection, UCLouvain) as MUCL41833 and the strain of *R.diaphanus* is registered as MUCL43196.

## 8.2 Preparation and maintenance of the *in vitro* culture systems[9]

### 8.2.1 Foreword concerning the *in vitro* culture systems and the experiment

In this work, we have made an experiment with *in vitro* culture systems to grow whole plants associated with AMF (see Figure 4 for an illustration of such a system). These systems have several advantages: they are free of any unwanted contaminant and allow an easy observation of the roots and AMF (and thus of the spores) since the medium is transparent.

### 8.2.2 Seed disinfection and germination

The *C.spectabilis* seeds were surface-sterilized during five minutes using a concentrated solution of sodium hypochlorite (with a chlorine concentration of 8%). After that treatment, the seeds were rinsed with sterilized distilled water during five minutes. The rinsing stage was repeated six times. The seeds were then placed in Petri dishes (92 mm of diameter and 16 mm of height) containing the Modified Strullu-Romand (MSR) medium (Declerck *et al*. 1998) without vitamins and sucrose. The MSR medium without vitamins and sucrose will be referred as $MSR^-$ medium. The exact composition of the MSR medium can be found in Annexe 1. Six seeds were placed per Petri dish. The Petri dishes were finally sealed, wrapped in aluminium to ensure total darkness, and placed in a phytotron (27 degrees Celsius, 80 per cent humidity) for a week (CESAMM Team 2019).

### 8.2.3 Transfer of the plantlets

One week after the disinfection of the seeds, the newly germinated plantlets were transferred in mono-compartment *in vitro* culture systems, under a laminar flow hood. Each system consisted of a Petri dish with a lateral hole under the top of the Petri dish, as shown in Figure

[9] By the term “system”, we mean the Petri dish plus the plant plus the AMF.

4. The Petri dishes were filled with 45 ml of $MSR^-$ medium. In each system, one plantlet was placed. The shoot system of each plantlet was placed across the hole so that the shoot was allowed to grow outside of the dish and the root system could develop inside the dish. This is also represented on the Figure 4. The plants were inoculated with a strain of AMF. The inoculum consisted of a piece of root-organ culture of transformed carrot root associated with the AMF strain on the MSR medium. The piece was chopped to separate the spores from the hyphae and stimulate new hyphal growth and then placed at proximity of the growing root tips of the plantlet (CESAMM Team 2019). Each inoculum thus consisted of colonized carrot root fragments and extra-radical hyphae bearing numerous spores. Two different strains representing different AMF species were used. As a result, some systems were inoculated with *R.irregularis* and others with *R.diaphanus*. A control, without AMF was also included in the experimental design.

*Figure 4 : Illustration of a mono-compartmented culture system. On this photograph, the plant is a potato. We can see the plant shoot growing outside of the Petri dish thanks to a side hole. The plant roots and the AMF develop inside of the contaminant-free dish (Voets et al. 2005)*

The Petri dishes were then sealed with *parafilm*™ and plastic film. Sterilized silicone grease was used to plaster the hole. All the systems were then placed in a phytotron (24 degrees Celsius, 80 per cent humidity) to allow the development of the plant and the AMF. The Petri dishes were stacked in piles in the phytotron and the position of the dishes and the piles was randomized each week. Black sheets placed between the Petri dishes kept the roots in dark conditions (CESAMM Team 2019).

Strangely, the root systems of the *C.spectabilis* and the AMF did not develop very well during the first weeks after their transfer in the *in vitro* culture systems. In order to help the development of the plant and the AMF, the systems were removed from the phytotron at 24 degrees Celsius and placed in a phytotron at 27 degrees. Following this transfer, the roots developed profusely as well as the AMF.

### 8.2.4 Maintenance of the systems

The Petri dishes were filled up again with $MSR^-$ medium each time the level of medium became too low. They were then opened under a laminar flow hood and newly prepared sterilized medium was added in all the dishes. The amount of medium added was variable and depended on the level of medium in each Petri dish. So, each Petri dish was filled up to more-or-less the height of the hole.

## 8.3 Data harvesting

### 8.3.1 Forming of the two groups

As previously mentioned, during the first weeks of growth the plants and the AMF developed slowly. After the transfer to a phytotron at 27 degrees, the extraradical mycelium developed profusely and produced a dense network of hyphae bearing thousands of spores. However, this development arose suddenly during academical activities (field practice in ecology) and during a COVID quarantine. After return to the lab, the hyphal network and the number of spores were already too high to be counted. The production of spores was already in the exponential phase. We decided to remove one half of the medium in each system to allow a new development from zero. The empty half of the Petri dish was then filled with fresh $MSR^-$ medium. The same area of medium was removed in each system. The removed area was chosen in function of the position of the roots. Indeed, we tried to remove the lowest amount of the root system as possible. The result of the operation can be seen on Figure 5, where the black line shows where the medium was cut. The photograph was taken a few weeks after the operation so that the roots had already colonised the removed area. The conclusion of this operation is that we obtain systems with two different “compartments”. Indeed, in the non-removed compartment, the roots are older and the density of spores is higher.

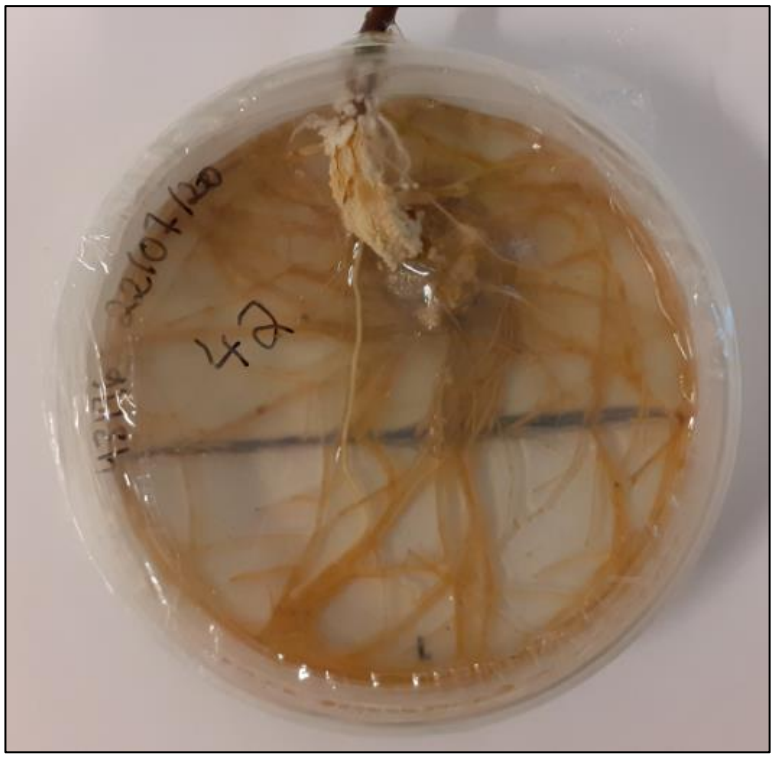


*Figure 5 : Illustration of the cutting operation of the medium. The black line made on the dish indicates where the medium was cut and delimits the two compartments. On the picture, the roots have already colonised the half dish where the medium was removed.*

After one week, the hyphae had already colonized the new medium of nearly all the systems. In some of them, it was already too dense to measure the hyphal length. But the counting of spores was feasible. The plates were then divided into two groups and placed in two phytotrons at two different temperatures (24 and 27 degrees Celsius). The division was made by qualitatively taking the size of the plants, the root density, the hyphal density and the spore density into account. We have proceeded as follows. We have done pairs of systems, trying to associate together two systems as similar as possible, considering the four factors. The control systems (without AMF) and the systems with the first and the second strains were considered separately. When the pairs were formed, one system of each pair was placed randomly in a phytotron. As a result, the controls and the two strains were distributed equally between the two temperature groups. One of the systems was not colonized by the AMF and was then removed from the experiment. Finally, another system was left without a partner. It was associated to one of the two temperature treatment randomly.

### 8.3.2 Spore counting

The spore counting started one week after the cutting of half of the content of the Petri dish. The count was done each week at the beginning of the experiment and then every other week at the end. There were two counters: T. Shewchenko and P-H. Jean. The first week, only P-H. Jean counted the spores. For the second and the next weeks, T. Shewchenko also counted spores. We counted each time the same systems to control the counter effect. The systems were also divided between the two counters so that each counter counted systems of each

group (temperature and AMF species). The exact dates of the counting operations as well as those of the main operations of the experiment are reported on a calendar, in Annexe 2.

Each counting was done as following. Each time we counted the spores of a system, we placed and pasted a grid of lines under the Petri dish (see Figure 6 for the grid of lines). We have counted the spores in the same six squares (1 square = 1 $cm^2$) of the grid, in the removed half of the medium, under a binocular. These six squares are the squares with an orange point on the Figure 6. The grid of lines was placed so that the cutting line was on the limit between the squares number 29, 30, 31, 32, etc. and the squares number 37, 38, 39, 40, etc. As a result, the six squares counted were always at the same place. These squares had the numbers 39, 40, 41, 42, 47 and 48. The counting was done with manual laboratory counting machines. We pressed the counting machine each time we saw a spore. For each count the sum of the spores in the six squares of a system was used as the value for the spore number.

### 8.3.3 Impact of the COVID crisis on the organisation of the experiment

Originally several strains of each AMF species should have been studied in order to measure the importance of the intraspecific variability in the response of the AM to the temperature increase. The experiment had to be repeated on several strains, in different sub-experiments. Other variables also had to be studied (P transfers, for example). Because of the COVID the lab has been closed from March to July and the previously prepared systems have been thrown away. This closure explains mainly why we have not had time to repeat the experiment with other strains of *R.irregularis* and *R.diaphanus*. The P transfers has not been studied because the systems of this other experiment have had too many contaminations. They were in big two-compartmented plates which are contaminated more easily.

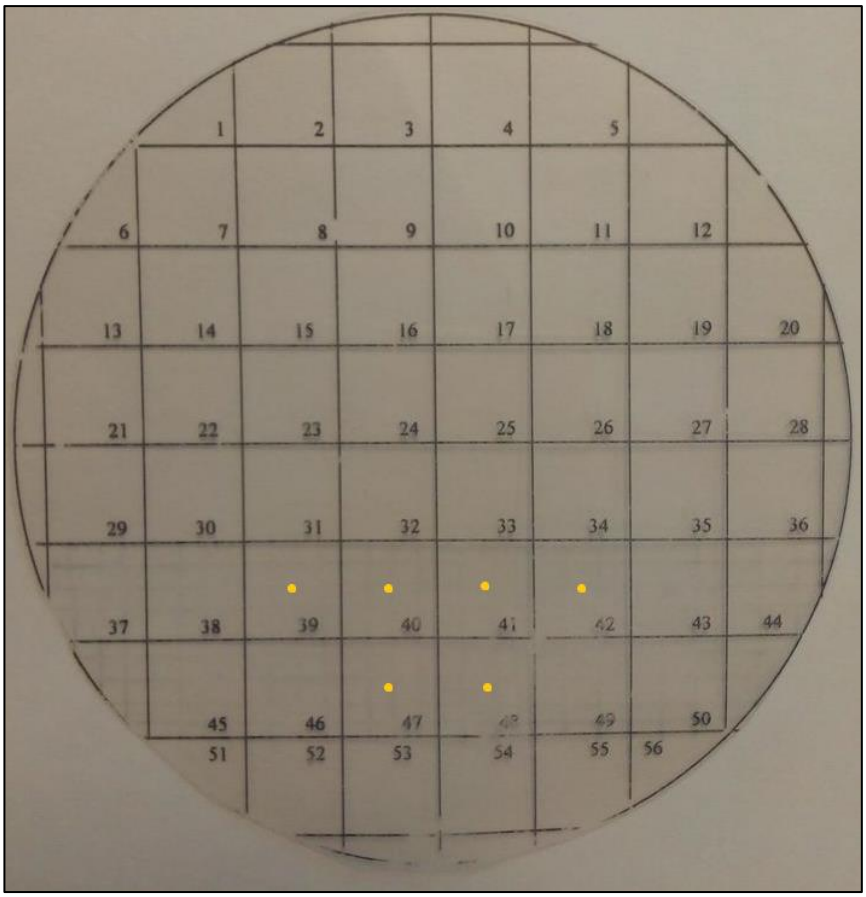


*Figure 6 : Illustration of the grid of lines used to count the spores in the Petri dishes. We can see the numbers in the squares. The six squares with the orange points are the squares used for the spore counting.*

### 8.3.4 Leaf counting

The photosynthetic area may impact the results of the spore production. Indeed, if some plants have more leaves, they may produce more hydrocarbons and send more of them to the AMF. As a result, the concerned systems may have more spores independently of the temperature treatment. Therefore, we decided to take the number of leaves with time into account. Each week, the number of leaves of each plant was counted. At the proximity of the apical meristem leaves were often small and compacted making the counting of young leaves difficult. We have then only considered leaves when their nerves became discernible.

## 8.4 Experimental design

### 8.4.1 Research questions

When the experimental design was made before the experiment two questions were defined:

1. What are the effects of an increased temperature on several parameters of the AMF?

2. What is the AMF intraspecific diversity in the AM response to an elevated temperature?

Because of the COVID crisis the number of AMF strains was reduced making the studying of the second question much harder. Besides the study has finally concerned the spore production. The hyphal density had to be studied but because of the cutting operation in the Petri dishes, the hyphae developed profusely. Indeed, several hyphae emerged from each cut hyphae after the operation making their counting impossible.

### 8.4.2 Statistical individuals and number of replicates

The statistical individuals are the experimental systems, i.e. one Petri dish with a plant inoculated or not with an AMF (controls do not have fungi). At the beginning of the experiment, 60 seeds were disinfected and 48 plantlets were transferred in individual experimental systems. Indeed, we had three groups (two species plus the control) and two temperatures per group for a total of six different treatments. We had then originally eight systems per group. Because of contaminations, the number of individuals decreased over the weeks.

### 8.4.3 Variables and response

The factors of interest are:

- The temperature: Controlled, qualitative (two levels: 24 and 27 degrees Celsius) and fixed factor
- The Species: Controlled, qualitative (two levels: *R.irregularis* and *R.diaphanus*) and random factor

But other factors have to be controlled: (1) the counter (Controlled, qualitative with two levels: T.Shewchenko and P-H.Jean, and random) and (2) the number of leaves (Uncontrolled, quantitative discrete).

The studied response is the estimation of the spore number in six square centimetres of the medium (Quantitative continuous, magnitude of several thousands of spores in the six squares, transformed (sqrt)[10]).

### 8.4.4 Control

Control plants were grown without AMF inoculation. They were originally needed to study other response-variables but with the changing in the experiment, the retained response

[10] This transformation has been decided a posteriori during the statistical analysis.

(spore production) could not be measured on systems without AMF. Control systems were thus not useful.

### 8.4.5 Randomization

Randomization has already been mentioned with the formation of the groups for the temperature treatments. It has also been said that the position of the plates in the phytotrons were randomized each week. Plants were also randomly assigned to the two counters. An order for the counting of the plates was also made randomly so that the systems were treated to the maximum on the same day of the week. So, some plates were always counted on Tuesday and some on Wednesday. In order to do that we have randomly assigned some plates of each treatment on Tuesday and on Wednesday. The order within a day was not always the same.

### 8.4.6 Summary of the experimental design

The Figure 7 is a summary of the original experimental design. The different factors (temperature, AMF species and AMF strains) are visible. The species and strains names are fictive. The experiment was separated into several sub-experiments. Because of time restriction, only the control plants and one strain for each species were studied.

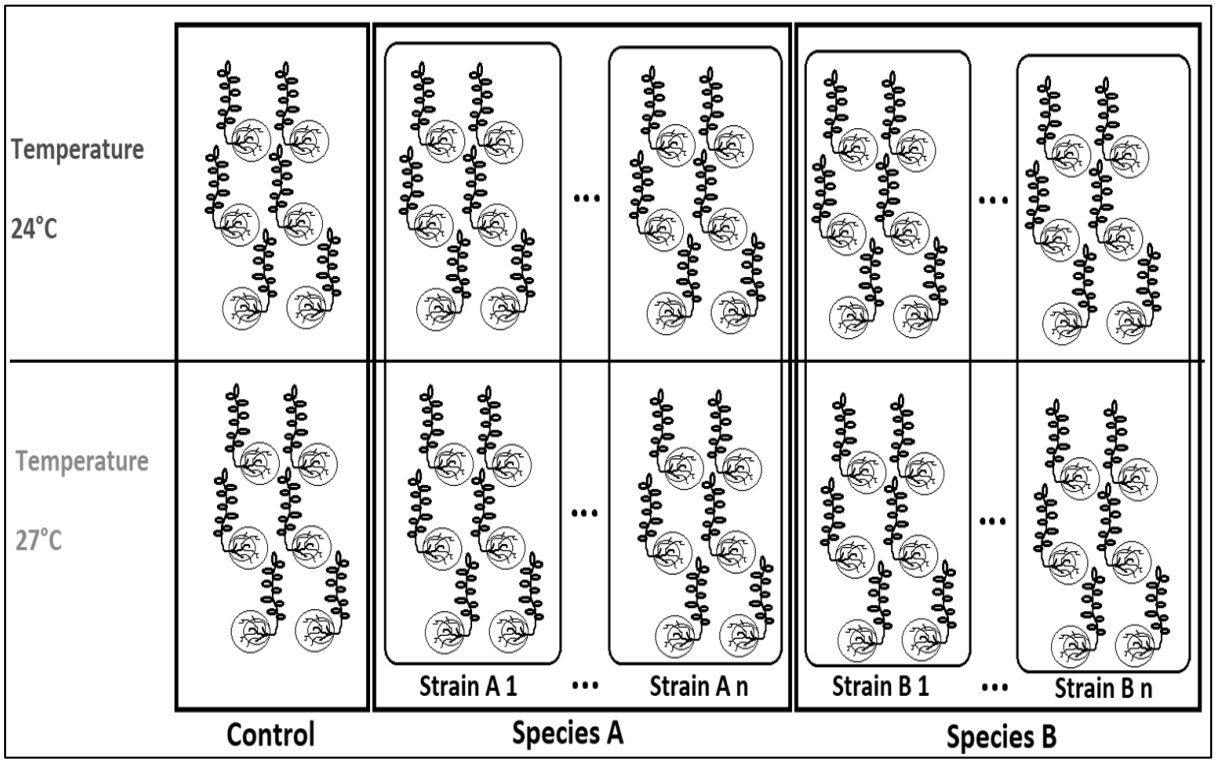


*Figure 7 : Visual summary of the original experimental design showing the studied systems and the different factors (temperature treatment, AMF species and AMF strains). The small pictures are experimental systems with a Petri dish and a plant growing outside of the dish thanks to a lateral hole.*

## 8.5 Statistical analyses

Firstly, data were analysed with descriptive statistics. Secondly, a model was adjusted to the data in order to measure the importance and the significance of the temperature effect with time. Additional effects such as interactions and quadratic effects were investigated. Finally, the assumptions of the model were then checked for the best suited model to ensure that it can be kept.

The model used is a linear mixed model with a random slope and a random intercept, as the example given below. Indeed, in this work there are repeated measures on the same systems. Mixed models are adapted to such grouped data.

$$y_i = (\beta_0 + a_j) + (\beta_1 + b_j).x_{i1} + \ldots + x_{ip} + \varepsilon_i$$

$$\text{Spores} = (\beta_0 + a_j) + (\beta_1 + b_j).Time + \beta_2.Species + \beta_3.Temperature + \varepsilon_i$$

with :

- $a_j \sim \text{Normal}(0, \sigma_a^2)$ and $\sigma_a^2 > 0$
- $b_j \sim \text{Normal}(0, \sigma_b^2)$ and $\sigma_b^2 > 0$
- $\varepsilon_i \sim \text{Normal}(0, \sigma^2)$

"Spores" is the spore number in the six squares considered per plate. "Time" is the week number. "Species" represents the AMF species. "Temperature" is the temperature treatment.

All statistical analyses were produced with the statistical software R with an α threshold of 0.05 and the data management was made in Excel.

# 9 Results

## 9.1 Descriptive statistics

Table 2 gives several descriptive statistics of our data: minimum, maximum, range, sum of non-missing values, median, mean, standard error on the mean, confidence interval of the mean, variance, standard deviation, coefficient of variation, number of observations, number of values, number of null values and number of missing values (Non Available values, NA). The number of values is the number of observations without the missing values. There are several missing values in the dataset because of the contaminations. Indeed, when a Petri dish was contaminated, it was suppressed from the experiment. The maximum is the maximum of spores in the six squares counted, not in the whole Petri dish. Indeed, because of the removing of a part of the medium, the system is made up of "two different compartments" with different densities of spores. As a result, an extrapolation of the spore density for the whole Petri dish with a count in only one compartment would be biased. However, as the "not removed" part of the dish had more spores than the removed one, we can give a minimum estimation for the spore production of the whole dish using the spore density in the six squares in the last count. The systems had in general from 2500 to 5000 spores in the six squares (see figure 12 for these numbers) and they had then from ± 20 800 to ± 41 600 spores in the whole Petri dish (50 squares), but these are underestimated values and the spore density was still increasing at the end of the experiment.

*Table 2 : Descriptive statistics of the dataset*

| **Statistic** | **Value** |
|---|---|
| Minimum | 0 |
| Maximum | 5890 |
| Range | 5890 |
| Sum | 151 119 |
| Median | 856 |
| Mean | 1536,56 |
| SE. Mean | 166,23 |
| CI. Mean | 329,88 |
| Variance | 2 735 609 |
| Std. Dev. | 1653,97 |
| Coeff. Var. | 1.08 |
| Number of observations | 114 |
| Number of values | 99 |
| Number of zeros | 22 |
| Number of NA | 15 |

The Figure 8 is a graph of the total spore production in the two temperature treatments. Graphically, there seems to be no global temperature effect. The Figure 9 shows the impact of the temperature within each AMF species. On this figure we see that the total spore

number is slightly higher in the 27-degrees treatment among the two AMF species considered. The difference between the two temperature treatments is higher in the group of plants inoculated with *R.irregularis*.

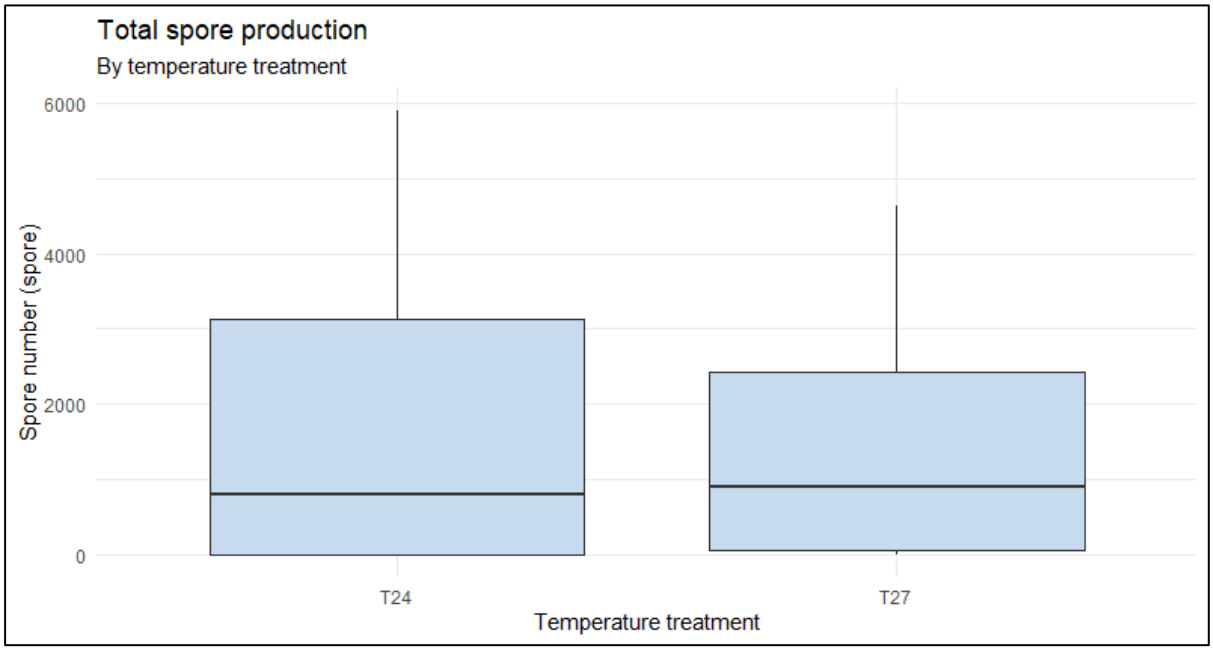


*Figure 8 : Graph of the total spore production in the 24- and the 27-degrees treatments.*

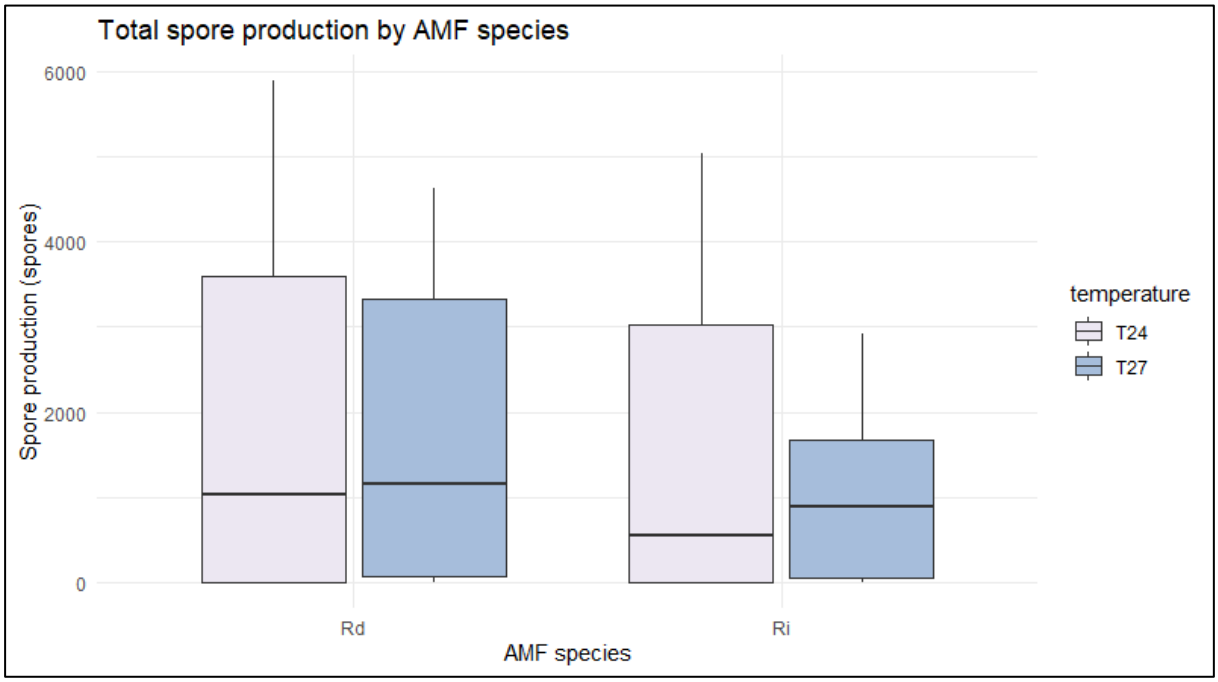


*Figure 9 : Graph of the spore production in the two AMF species with details for each temperature treatment. "Rd" means Rhizophagus diaphanus and "Ri" means Rhizophagus irregularis.*

The Figure 10 shows the spore number evolution with time per temperature treatment. In comparison with the boxplot of the Figure 8, the Figure 10 shows the time effect. On it, we can see that the mean spore production is higher in the 27-degrees treatment during the first

four weeks. After this point, the mean spore production of the 24-degrees treatment becomes higher and it remains higher until the end of the experiment.

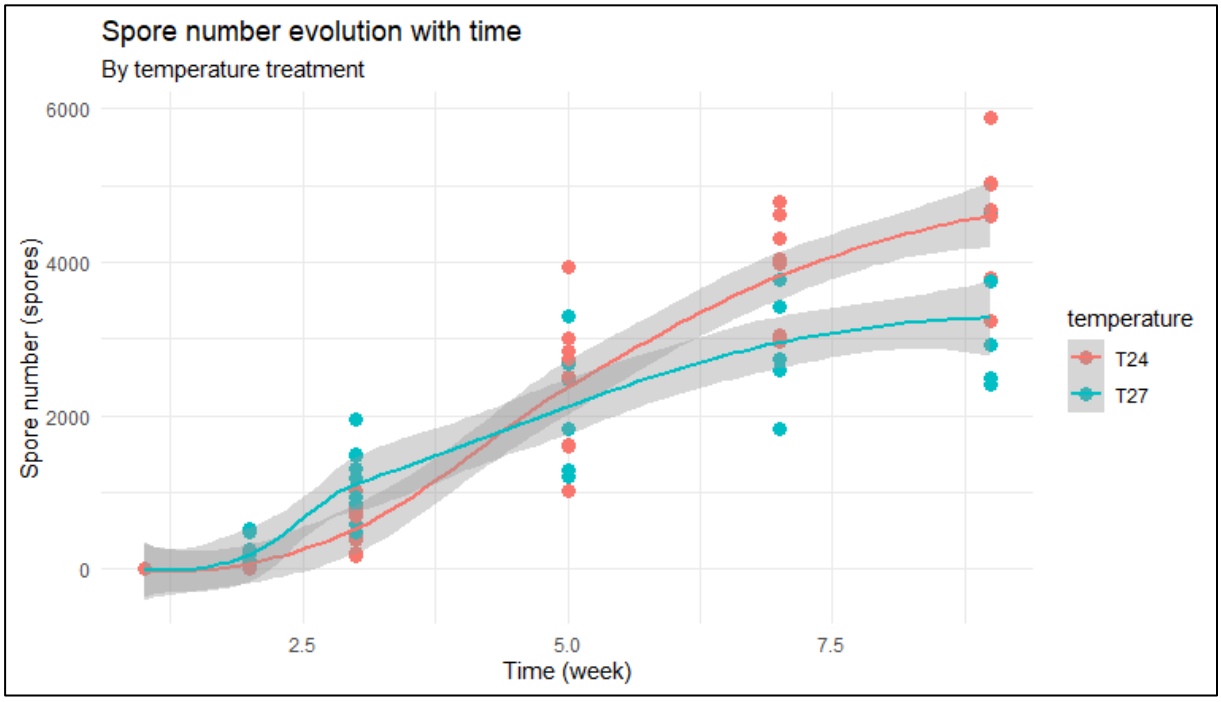


*Figure 10 : Graph of the evolution of the number of spores in the two temperature treatments.*

If we look at the effect of the temperature with time within the AMF species (Figure 11) we see a similar pattern for the two species. For each species, the mean spore production is higher at the beginning of the experiment for the 27-degrees treatment and higher at the end of the experiment for the 24-degrees treatment. The difference between the two temperature treatments is stronger for the group of plants inoculated with *R.irregularis*. This difference is consistent with the boxplot of the Figure 9.

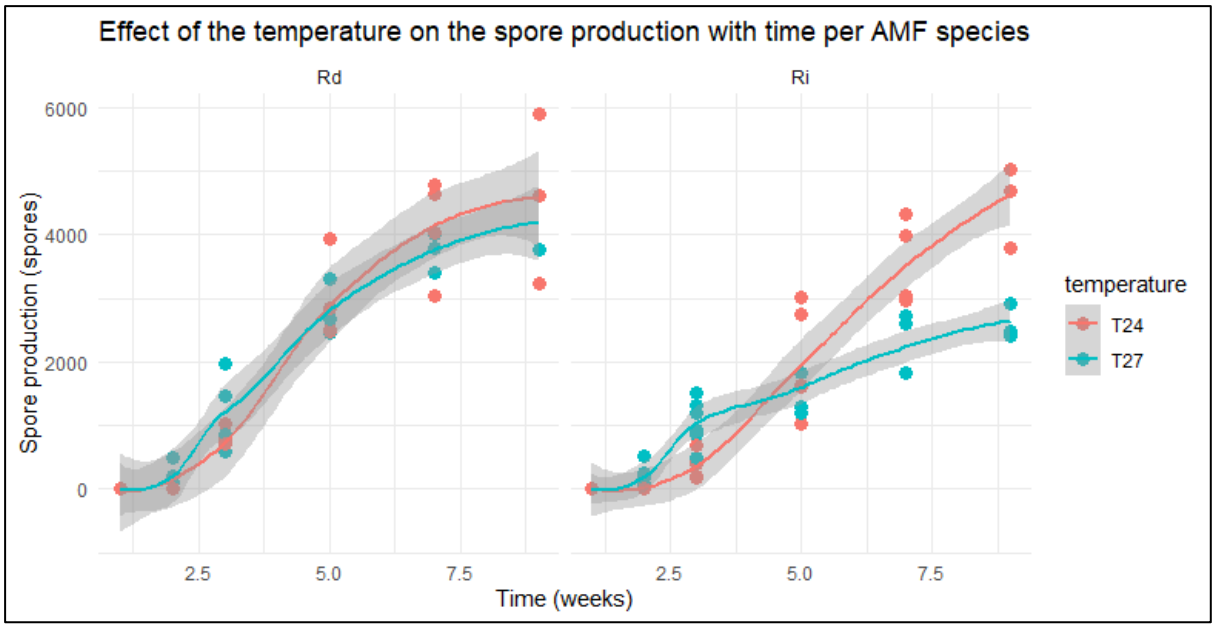


*Figure 11 : Impact of the temperature on the spore production with time, for each AMF species. "Rd" means Rhizophagus diaphanus and "Ri" means Rhizophagus irregularis.*

The Figure 12 shows the spore production of each experimental system during the experiment. On this figure we can easily see the sporulation value of each count for each system. On this figure we also see a difference between the variance at the beginning of the experiment (low variance) and at the middle and the end of the experiment (higher variance). The Figure 13 shows the same information than the Figure 12, but there is one graph for each system. We can then better see the curve shape of each system and its sporulation dynamic. We see better the heterogeneity in the sporulation dynamic between the different systems on the Figure 13 than on the Figure 12. As an example of this heterogeneity in the sporulation dynamic, at the end of the experiment some systems seem to have reached a steady state (ex: systems number 33, 37, 42) while some other systems have a spore number still increasing (ex: systems number 26, 30 and 40). We can also see on this Figure 13 that some curves do not reach the left side of their graph. This is because of the contaminations. When a Petri dish was contaminated, it has been removed from the experiment. Indeed, it was often difficult to count the spores in the contaminated dishes because of the fungal or the bacterial development. Besides, because of the bacterial/fungal invader there was competition/interactions with the AMF making the comparison with non-contaminated systems unrealistic. As a result, the curve of the spore production with time of the contaminated dishes ceases earlier than the end of the experiment. In the Annexe 3, there is a bigger graph of the spore production with time for each experimental system.

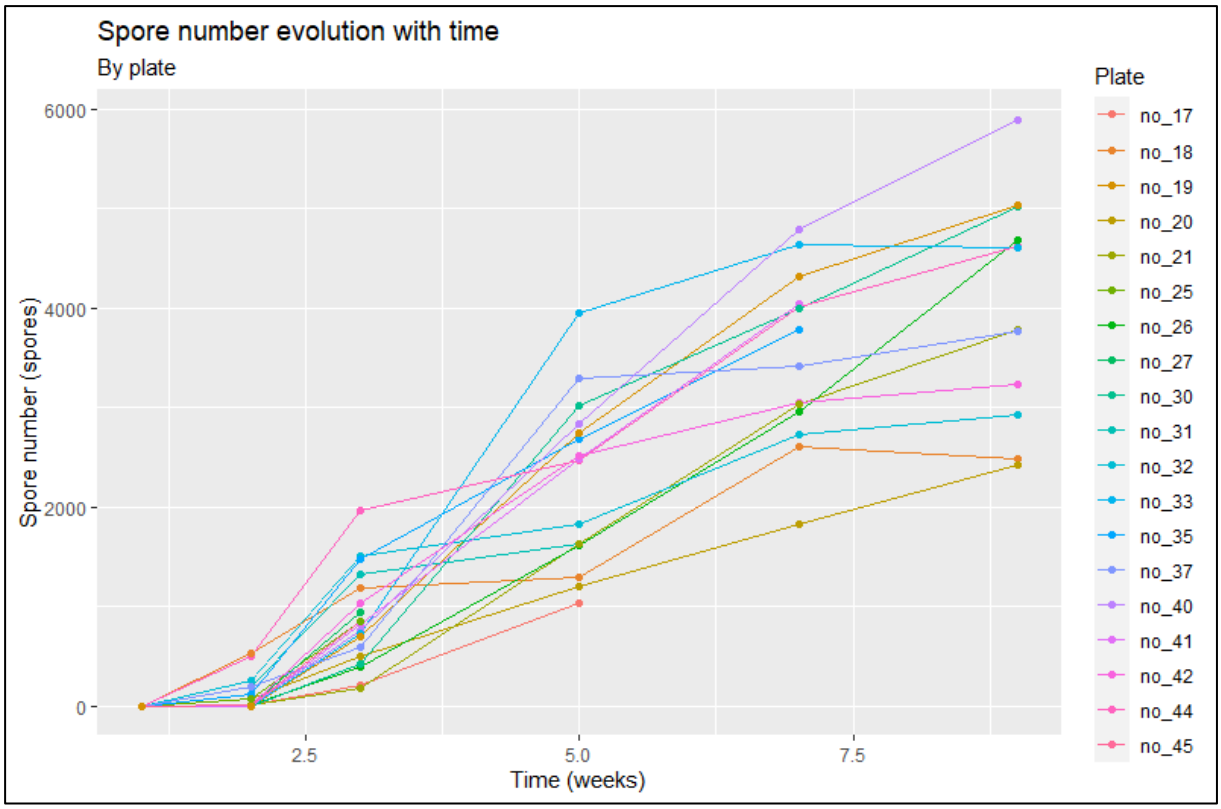


*Figure 12 : Spore production of each experimental system during the experiment.*

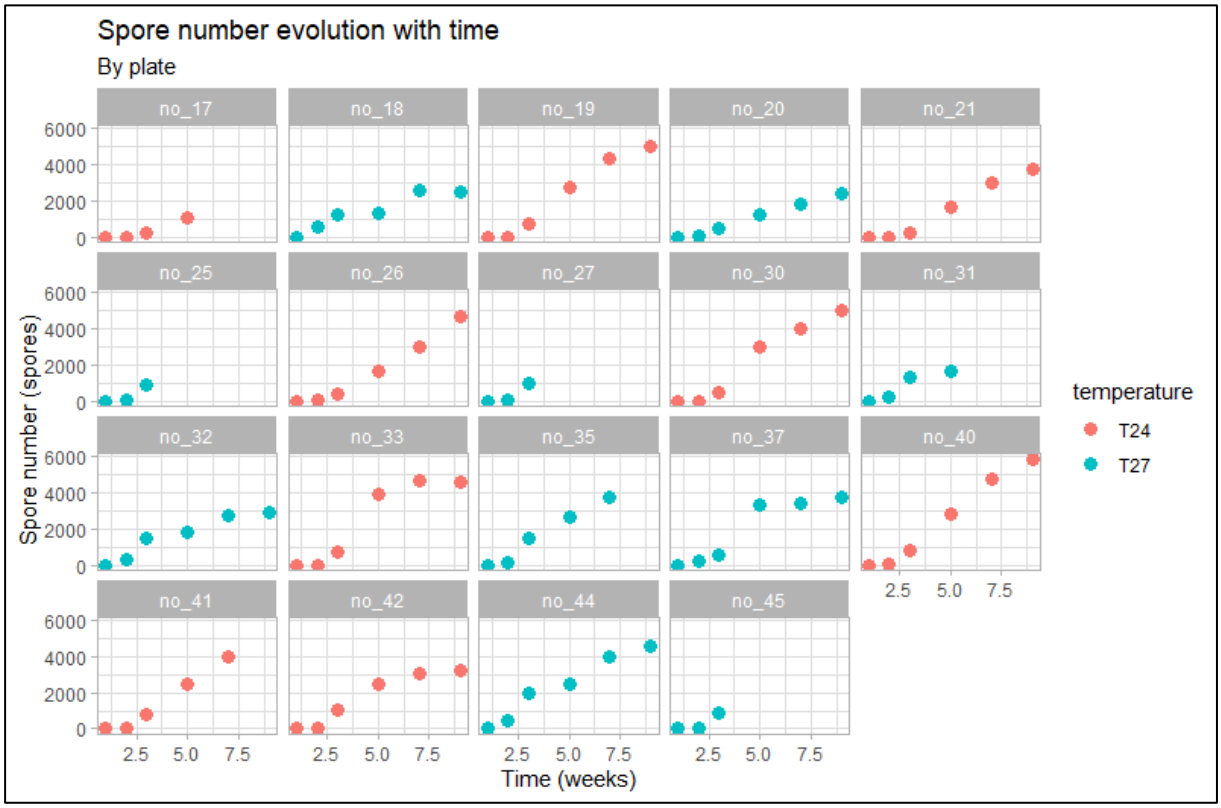


*Figure 13 : Separated graphs of the spore production by each plate, during the experiment.*

## 9.2 Statistical modelling

### 9.2.1 Akaike Information Criterion (AIC) of the different models tested

In the Table 3, the different models entered in R and their AIC are reported. We can see that the models with transformed data (squared root, sqrt) have a far lower AIC. This transformation has been chosen during the checking of the conditions because it makes the data distribution normal. The last model has been kept because its AIC is the lowest: this model is the best compromise between model quality (having a model fitted to the data as well as possible) and complexity (having a model with not too much parameters and which can be interpreted easily) (Zuur *et al*. 2017).

*Table 3 : Table of the different models tested in R and their Akaike Information Criterion (AIC).*

| **Model in R** | **AIC** |
|---|---|
| lmer(Spores ~ Time + (Time\|Plate)) | 1507 |
| lmer(Spores~Time + Temperature + (Time\|Plate)) | 1496 |
| lmer(Spores ~ Time + Species + (Time\|Plate)) | 1494 |
| lmer(Spores ~ Time + Species + Temperature + (Time\|Plate)) | 1483 |
| lmer(sqrt(Spores) ~ Time + Species + Temperature + (Time\|Plate)) | 727 |
| lmer(sqrt(Spores) ~ Time + $Time^2$ + Species + Temperature + (Time\|Plate)) | 673 |
| lmer(sqrt(Spores) ~ Time*Temperature + $Time^2$ + Species + (Time\|Plate)) (= selected model) | 666 |

### 9.2.2 Details of the selected model

The equation on the selected model is:

$$\sqrt{Spores} = (-22{,}88 + a_j) + (19{,}34 + b_j).Time - 1{,}02.(Time)^2 - 3{,}66.I(R.irregularis) + 9{,}98.I(T27) - 2{,}15.Time.I(T27) + \varepsilon_i$$

With:

$$a_j \sim N(0, (0{,}6582)^2)$$

$$b_j \sim N(0, (0{,}7336)^2)$$

$$\varepsilon_i \sim N(0, (6{,}2080)^2)$$

$$cor(a_j, b_j) = -1{,}00$$

$$j = 1, \dots, 19$$
$$i = 1, \dots, 99$$

In the equation, I(*R.irregularis*) = 1 for systems inoculated with this strain and I(*R.irregularis*) = 0 for systems inoculated with the other strain (*R.diaphanus*). Similarly, I(T27) = 1 for systems of the 27-degrees treatment and I(T27) = 0 for systems of the 24-degrees treatment. In this equation the time is calculated in weeks. The confidence intervals of each parameter are given in the Table 4.

*Table 4: Table of the confidence interval for each parameter estimated.*

| **Parameter** | **2,5%** | **97,5%** |
|---|---|---|
| Intercept | -27,69 | -17,88 |
| Time | 17,19 | 21,50 |
| $Time^2$ | -1,22 | -0,81 |
| Species (*R.irregularis*) | -6,85 | -0,56 |
| Temperature (27 degrees) | 5,47 | 14,57 |
| Time*Temperature (27 degrees) | -3,33 | -0,88 |
| Standard deviance $a_j$ | 0,00 | -0,46 |
| Standard deviance. $b_j$ | 0,17 | 1,34 |
| Standard deviance. $\varepsilon_i$ | 5,27 | 7,15 |
| Correlation ($a_j$,$b_j$) | -1,00 | 1,00 |

All the fixed effects of the selected model are significant (p-value < 0,01 except for the AMF species with a p-value < 0,05). The temperature and the AMF species have then a significant impact on the spore number. Besides, the time modulates the temperature effect and has a quadratic effect. The R-output of the selected model is given in the Annexe 4, at the end of this document. The R-output with the coefficients of the curve adjusted to each system in the selected model is also available in the Annexe 4. An interaction between the AMF species and the temperature has also been tested but this interaction was not significant (p-value > 0,05). The R-output of this other model is available in the Annexe 5. The temperature does not impact the two strains in a different way significantly.

The Figure 14 is a graphical visualisation of the selected model. It illustrates the spore production with time in function of the temperature treatment. This pattern (with a better mean for the spore production in the 27-degrees treatment at the beginning and a better mean for the 24-degrees treatment after a few weeks) has already been observed on the Figures 10 and 11.

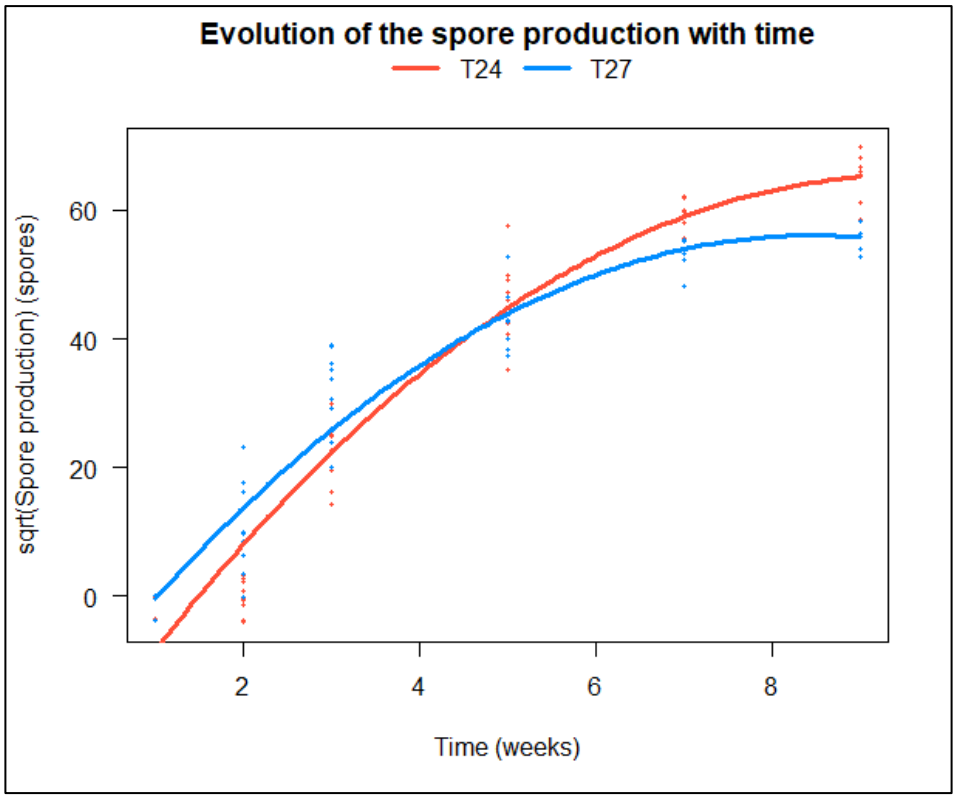


*Figure 14 : Graphical visualisation of the selected model in function of the temperature treatment.*

### 9.2.3 Testing the assumptions of the model

Two conditions are required to use a linear model: (1) the distribution of the residuals must be normal and (2) the variance must be homogeneous (homoscedasticity).

#### *9.2.3.1 Normality of the data*

The Figure 15 shows the QQ-plot of the selected model. This graph is not perfect, but it is acceptable. Besides, a Shapiro-Wilk test of the residuals of the model is not significant (p-value = 0,65). The data can then be considered as non-significantly different from the normal distribution.

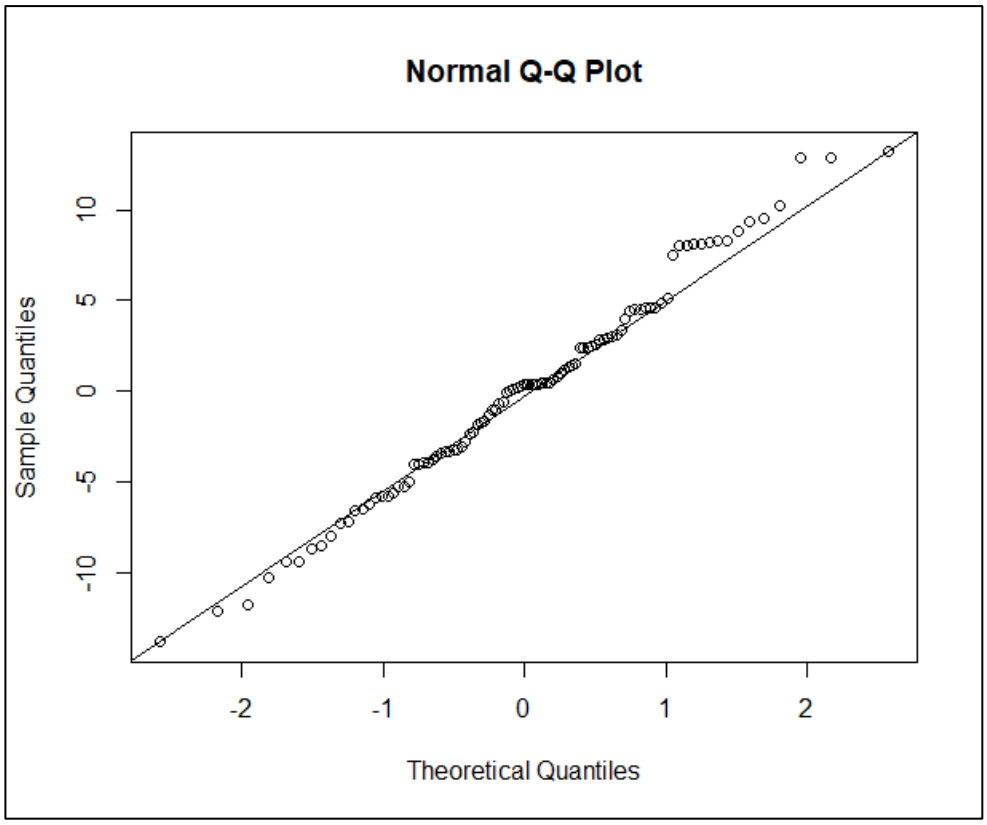


*Figure 15 : QQ-plot of the selected model*

### *9.2.3.2 Homogeneity of the variance*

Concerning the homogeneity of the variance, the Figure 16 shows the residuals of the model in function of the fitted values. Once again, the graph is not perfect. Some values on the left of the graph are problematic. It is mainly because all the plates had zero spores in the counted squares at the beginning of the experiment (the variance was low), but with the time the variance between the plates increased. However, the graph is quite acceptable. Indeed, we can see that the variance rapidly stabilizes. Beyond 20, the variance does not increase any more. The difference in the variance between the beginning and the end of the experiment is well illustrated on the Figure 12.

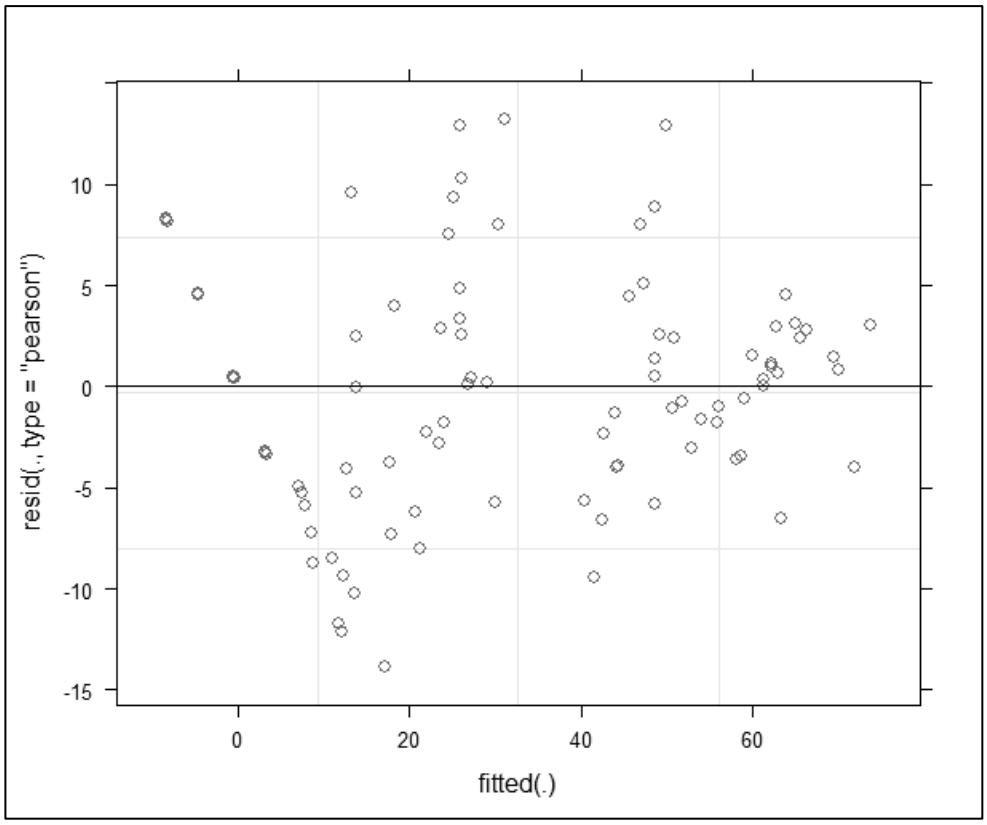


*Figure 16 : Graph of the residuals versus the fitted values*

### 9.2.4 Adequacy of the mixed model

There are many types of mixed models in R and this type (x + (x|g)) is adapted for models with a correlation between the random slope and the random intercept (Bates *et al.* 2015). We are precisely in this case, as it can be seen on the figure 17: the higher the intercept of the curve of each system, the lower its random slope.

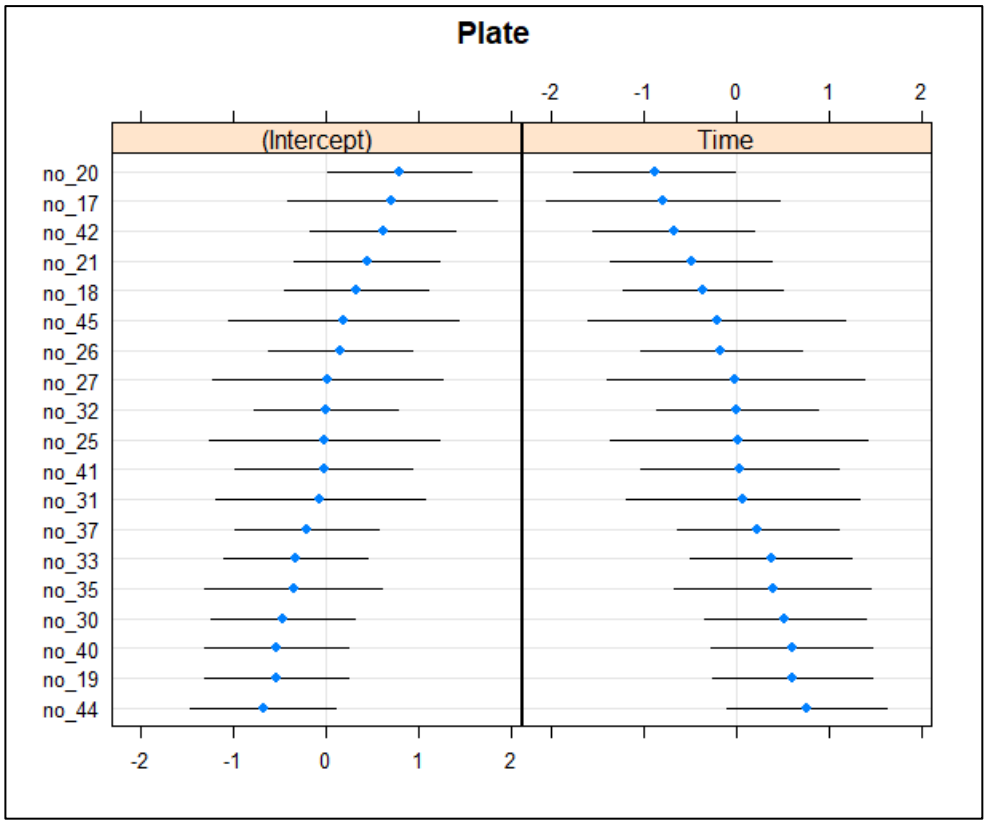


*Figure 17 : Graph showing the correlation between the random intercept and the random slope in the selected model. When the random intercept of the curve of one system is low, the random slope is high and vice versa.*

### 9.2.5 Detection of other effects

#### *9.2.5.1 Effect of the counter*

A model taking the counter into account has been adjusted:

$$\sqrt{spores} = Time * Counter + Time^2 + (Time|Plate)$$

On average the spore numbers reported for the plates counted by PH. Jean are higher than the numbers reported by T.Shewchenko. But neither the counter effect nor its interaction with the time are significant (p-value > 0,1). The R-output of this model is available in the Annexe 6, at the end of this document.

#### *9.2.5.2 Effect of the number of leaves*

As it has been said earlier the number of leaves has to be considered to be sure the photosynthetic area does not impact the spore production. The Figure 18 shows the evolution of the leaf number in the different plants in function of the time during the experiment. The temperature treatments are represented with two different colours. We can see on this graph that the systems of the 27-degrees treatment have lost more leaves than the systems of the 24-degrees treatment during the experiment. However, in two linear mixed models taking

respectively (1) the number of leaves plus its interaction with time and (2) the number of leaves and its interaction with temperature, the leaf effect is never significant (p-value > 0.05). The R-outputs with the details of these models are available in the Annexe 7. Some systems of the two temperature treatments are visible on the Figure 19. The losing of leaves is visible on both sides but especially on the plants of the 27-degrees plants (right of the figure). Be careful, the leaves of the plants of the 27-degrees are "closed" because the photograph has been taken when their growth chamber was in the night mode. The leaves of the plants of the 24-degrees treatment seem more spread out but it is because their growth chamber was in the day mode when the photograph has been taken. The two photographs have been taken at the same time but there was a lag between day-night program of the two growth chambers.

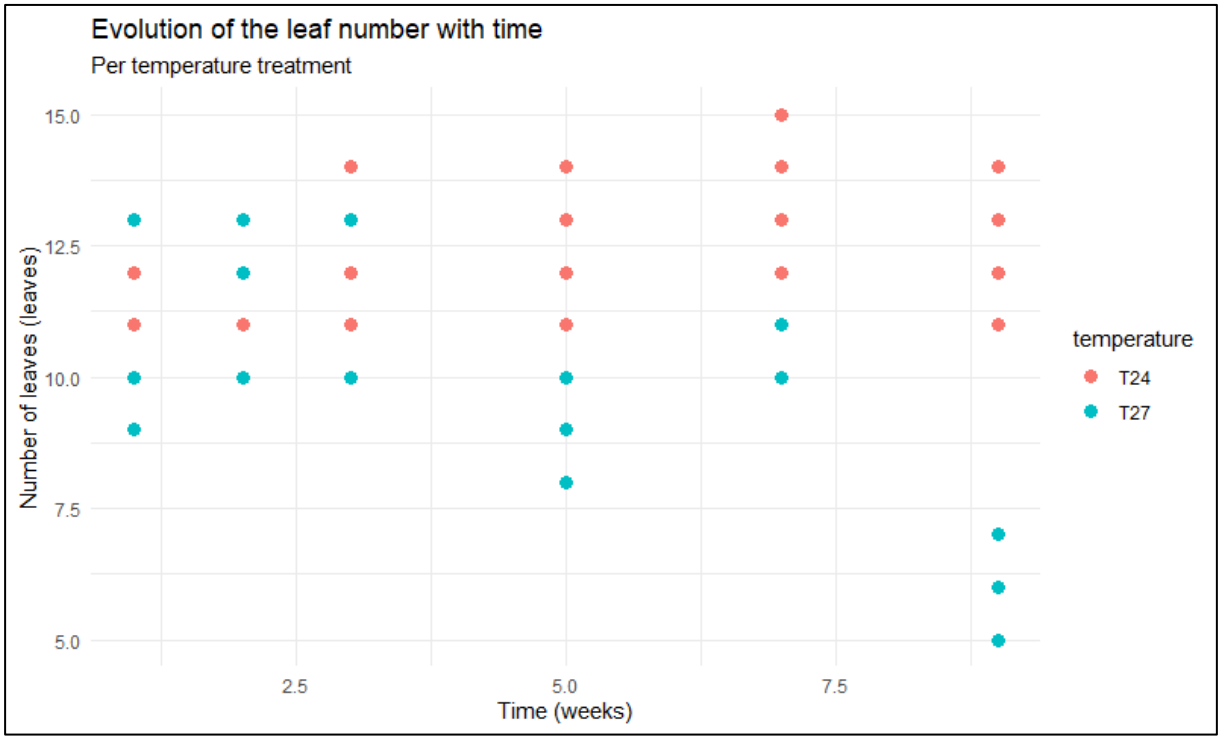


*Figure 18 : Graph of the evolution of the leave number with time per temperature treatment.*

*Figure 19 : Photograph of 24-degrees systems (left) and 27-degrees systems (right). We can see the smaller number of leaves on the plants of the 27-degrees treatment and the leaf scars.*

# 10 Discussion

**Importance of the effects**: In the chosen model (smallest Akaike Information Criterion), there is a significant effect of the temperature increase on the spore production. At the beginning of the experiment, the temperature increase had a positive effect on the sporulation. Around the middle of the experiment, the temperature effect became negative. This effect is quite important. According to the selected model, plants inoculated with *R.diaphanus* in a growth chamber at 24-degrees will have on average 910 additional spores in the six counted squares after eight weeks. If these plants were inoculated with *R.irregularis*, they would have on average 855 additional spores after eight weeks, in comparison with the 27-degrees treatment. The p-value is low enough to be confident in the significance of the temperature effect (p-value < 0,01). As said in the state of art both positive and negative effects of temperature increase on spore production have been reported in the literature. The present results must be added to those with a negative impact with the warning that the strains are not replicated. The two chosen strains have maybe a particular sensitivity to the temperature. In the articles cited in the state of art, we can find experiments in the fields and experiments using *in vitro* root organ cultures. In these articles, Mei *et al.* (2019a) isolated spores from the ground with sieving and centrifugation and counted them under a microscope while Costa *et al.* (2013) obtained and counted them the way we did. This work, on the other hand, is a manipulative experiment realised on *in vitro* systems with whole plants (not only the roots). This difference deserves to be underlined because "triangulation", i.e. studying a subject with a variety of methods, plays an important role in science to study one question and draw robust conclusions (Munafo *et al.* 2018).

Concerning the AMF species effect, the effect is smaller. For instance in the model, eight weeks after inoculation, plates inoculated with *R.Diaphanus* would have 475 additional spores in comparison with plates inoculated with *R.irregularis*, at 24 degrees Celsius. This effect is also significant (p-value < 0,05). Nevertheless, the temperature does not impact the two strains differently in a significant way (p-value of the interaction between temperature and the AMF species > 0,05). A similar pattern with an initial beneficial effect of higher temperature and a later detrimental effect is observed among the two AMF strains. As we have said earlier the original objective of the experiment was to study several strains from several species. This would have helped us to study the importance of intraspecific diversity. The time restriction and the resulting number of strains studied do not allow us to draw a conclusion on this aspect, which is regrettable given the absence of results concerning this point in the literature.

**Validity of the model**: The model indicates a significant temperature effect but is the model valid? Several aspects need to be discussed. There seems to be a small impact of the counter, but it is not significant. This is good because a counter effect could impact the reproducibility of the experiment. Besides, precautions were taken in order to avoid confusion between the counter effect and the other effects. Indeed, each counter has counted spores in systems of each treatment (temperature and species).

Among the bias, an underestimation of the spore number may be reported because of the root presence. Roots are too opaque to see and count the spores above and below them. The spore number is then certainly underestimated in the squares where roots were present. Taking the root density into account would have been a good idea. Taking six squares not close

to each other (cf. Figure 6) would also have been better by avoiding spatial correlation. Indeed, a root in one square has a high chance to be also present in the nearby squares.

Still in the framework of detectability of spores, the detectability of spores has also probably changed during the experiment with the increasing aptitude of the counters. However, this increase has probably impacted the different temperature treatments in the same way because each scientist counted the different treatments during each counting session. Besides the detectability of the spores greatly changed from one week to another because of the change in spore density. Counting from zero to one hundred spores per square-centimetre during the first week and up to 1800 spores (the highest record) per square centimetre during the last weeks is not the same exercise. The probability of counting a spore two times or to forget a spore was higher in the last weeks. But here too, we can reasonably assume that the change in spore detectability has impacted the two temperature treatments in the same way. Indeed, the two treatments had throughout the experiment roughly the same spore density.

Is there an impact of another abiotic factor? We have already said that the effect of the leaf number on the spore production is not significant with our data, as well as its interaction with time or temperature. However, the graphical analysis of the number of leaves per plant shows a clear difference between the two temperature treatments (Figure 18). On this figure we see that the plants of the 27-degrees treatment have lost more leaves during the experiment. This defoliation may be caused by a water stress and then be an indication of a greater lack of access to water in the 27-degrees treatment. The Petri dishes were filled up with $MSR^-$ medium when the medium level became low (often after two weeks - two weeks and a half, in general). Then the Petri dishes were fully filled (with 40-50 ml of medium). The 27- degrees systems have probably suffered a bit more of this process during the days before each feeding operation, when the medium level was lower. Maybe filling them with a bit less medium but more often would have been better for the 27-degrees plants. They would have never been under a certain level of medium and the temperature treatment would then have been differentiated from an effect of the water access. However, opening more often the Petri dishes to feed the plants also means having more contaminations and then less data, each opening of the sterile Petri dishes being a risk of contamination. It would have necessitated more systems and then more time (counting their spores and feeding them), more material and more space. We are in the classical trade-off between feasibility of the experiment (ability to do it) and human/material cost of an experimental design (figure 20): a better designed experiment often involves more resources (time-people-material) and has then a lower feasibility. In this case the higher rate of contaminations due to a lack of experience for *in vitro* culture in a young scientist was a critical parameter. In conclusion there may be a confusion between the temperature effect and an effect of the access to the water. A temperature increase without a water stress would maybe have no impact or a positive impact on the spore production. An experiment realized in the lab on the impact of the water access on several AMF parameters (including the sporulation) indicates indeed a negative impact of a smaller access to water on the spore production (Le Pioufle *et al.* 2018). This experiment has been done in the same *in vitro* conditions and with the same AMF strain (*R.irregularis*, MUCL41833) than in this work. Better differentiate the water access and the temperature seems then an important aspect for future experiment on the temperature impact on AMF sporulation. Finally, it must be underlined that the models taking the leaves number into account all show a non-significant effect for this parameter, but the leaf number is maybe a bad indicator of the water stress. *A posteriori*, we could say that testing the stomata opening level or check

the foliar temperature with infra-red thermography would have been useful to detect a water stress.

Moreover, the presence of a water stress offers several hypotheses to explain the decrease of the spore production in the systems of the 27-degrees treatment. A water deficit can (1) increase the production of Reactive Oxygen Species (ROS), (2) inhibit photosynthesis or (3) cause an osmotic stress into the plants, for example (Taiz *et al.* 2010). In the last situation, when the water potential of the soil (here the medium) decreases because of a water deficit, plants have to diminish their own water potential to continue to absorb water (Taiz *et al.* 2010). As a result, besides the diminution of the photosynthetic area because of leaf abscission, plants of the 27-degrees treatment have maybe produced less spores because of one (or more) of these three problems. In the first case the 27-degrees plants would have spent more C and energy to produce enzymes to transform the ROS (catalase, superoxide dismutase, etc.). In the second case, the inhibition of the photosynthesis would simply have reduced the production of photosynthetic products. In the third situation, they would have spent more C and energy to produce osmolytes, small organic compounds osmotically active and compatible with the normal metabolism such as proline, sorbitol or glycine betaine, in order to diminish the water potential of the plant cells (Taiz *et al.* 2010). Further biochemical and physical analyses would have been required to test such hypotheses.

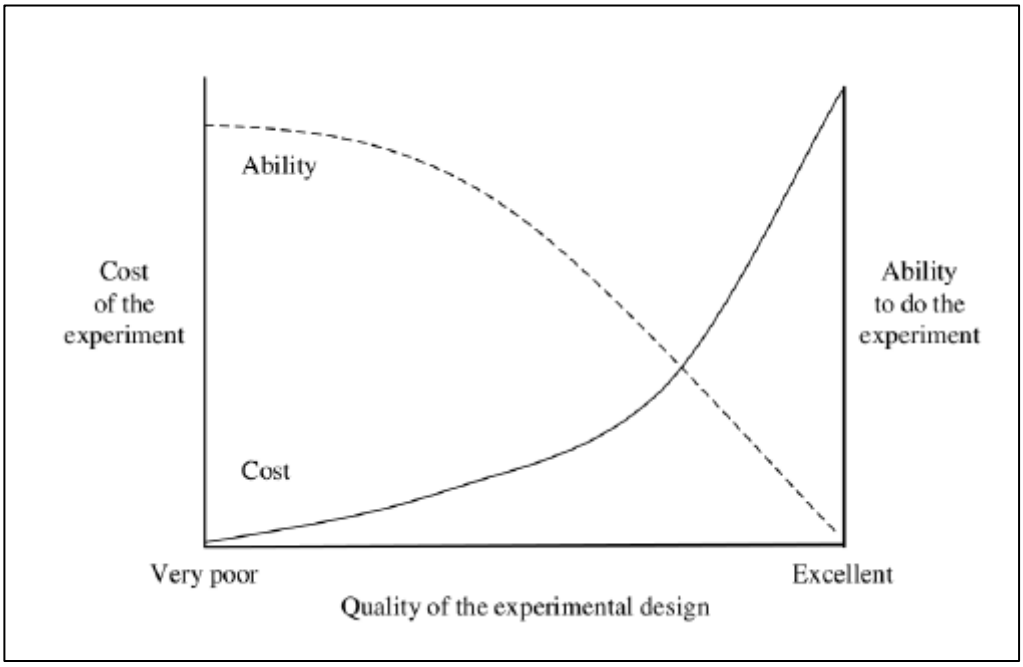


*Figure 20 : Graph illustrating the trade-off between cost (time, money, people and space) and feasibility in the designing of experiments (McKillup 2005)*

Is the model realistic? Does it make biological sense? In our model, a linear mixed model, a straight line has been adjusted to the spore data of each system. This point has to be discussed. The curve of the spore production by AMF usually follows a "S-shape", with an exponential phase and a final steady state (Declerck *et al.* 2001). However, a linear mixed model with a straight line is still acceptable for our data because for many of the plates a S-shape curve is not evident, especially for the plates with only two-three points (contaminated

ones). Scientists ignorant of the existence of the S-shape pattern would hardly guess it from many of the plates. This is the case for the plate number 26, 27, 35, 45, 25, 18, 40 and 41 (Figure 13). S-shaped model could be more accurate, but it would also have needed more points, especially more frequent points in order to detect accurately the inflection point of the S-curves. Indeed because of the spore explosion in the plates we have shifted to a rhythm of one count per two weeks causing relatively big gaps between points. However, more frequent points (smaller gaps) and a longer experiment (to reach the steady phase of the S-pattern) would also have increased the investment raising one more time the question of the position in the feasibility/cost trade-off.

**Independence of the statistical units:** One frequent problem in experimental design is pseudoreplication. Before the COVID crisis the experimental design involved the study of several strains per AMF species. Because of the limited time two species have been studied with one strain per species. Are the resulting experimental units truly independent? The question is a bit difficult. Because the inoculums coming from the same AMF strain are ramets from the same genet (they are clones) we could ask if the strains would not be the real replicate. However, the statistical unit (each system) is made up of a fungus but also of a plant. As a result, the different statistical units were all different because the plants are not clones. The plates inoculated with the same strain are not independent but this link between them is indicated in the model (presence of a species factor). Moreover, a great variability is observed between the spore production of the systems inoculated with one AMF strain. For instance for the last count the plates 32 and 19 (both inoculated with *R.irregularis* and placed at 27 degrees Celsius) had respectively 2921 and 5027 spores in the six counted squares. Seeing this variation, the replication within the strain is useful. It will have to be taken into account in the designing of a future similar experiment, as well as the multiplication of the strain number. These two levels (within the strains and between the strains of one species) are necessary to understand the importance of intraspecific diversity into the response of the AMF to an increase in temperature. Two-three species with several strains in each and with several plants inoculated per strain would represent an ideal experiment but it represents a high quantity of work, mainly because of the spore counting.

# 11 Conclusion and perspectives

In conclusion, the model used has allowed us to detect a significant impact of a higher temperature on the spore production of two AMF strains from different species inoculated to plants of *C.spectabilis*. During the first four weeks, the temperature increase had a positive impact and after that point, the sporulation of AMF at lower temperature became higher. In addition to a temperature effect, the diminution of the photosynthetic area or a secondary effect of a water deficit could explain the lower spore production of the 27-degrees systems. The temperature increase does not impact significatively the two species in a different manner, but the small number of species studied do not allow us to draw a conclusion on the interspecific diversity in the response to temperature increase. An intraspecific diversity was observed between the plants inoculated with the same AMF strain but here too, the number of strains per species studied avoid the studying of the diversity between strains of the same AMF species.

Several points could be ameliorated in a future similar experiment. The systems may be fed more often with smaller amounts of medium in order to be sure that the plants always have enough water (more constant medium level). This may help to distinguish between the effect of the temperature and an effect of the access to water. Several ways to control the existence of a water stress have been proposed. The root density could also be put in the model to take into account the fact that the roots occult the presence of spores in the counted area. A S-shape model could also ameliorate the biological realism of the modelling. More points would be useful for such an adaptation of the model.

Finally, the trade-off between feasibility and cost in the experimental design has been an important aspect of this work. Counting up to 1800 spores in a square centimetre with a binocular takes a lot of time and it is emotionally trying. Ameliorating the quality of the experimental design often involves studying more plates and counting more spores. It would then be interesting to find a way to count the spores more easily or automatically. Automatic or semi-automatic tools already exist to measure the hyphal length for instance. The software HyLength, for example, allows the measuring of the hyphal length with results in *in vitro* cultures of *R.irregularis* highly similar to manual measures (Cardini *et al.* 2020). HyLength permits a great gain in time in comparison with manual counting and it ameliorates the reproducibility of the experiment. Finding a similar way to study the spore density would certainly allow great progress in the comprehension of the impact of the temperature and the modulation of the AM response by the AMF intraspecific diversity.

# 12 Sources


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# 13 Annexes

## 13.1 Annexe 1: Modified Strullu-Romand (MSR) medium composition

*Table 5 : Composition in vitamins and minerals of the Modified Strullu-Romand (MSR) medium and the Minimal (M) medium. In order to have MSR-, the vitamins have to be removed. (CESAMM Team 2019).*

| | *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 |

## 13.2 Annexe 2: Calendar of the experiment

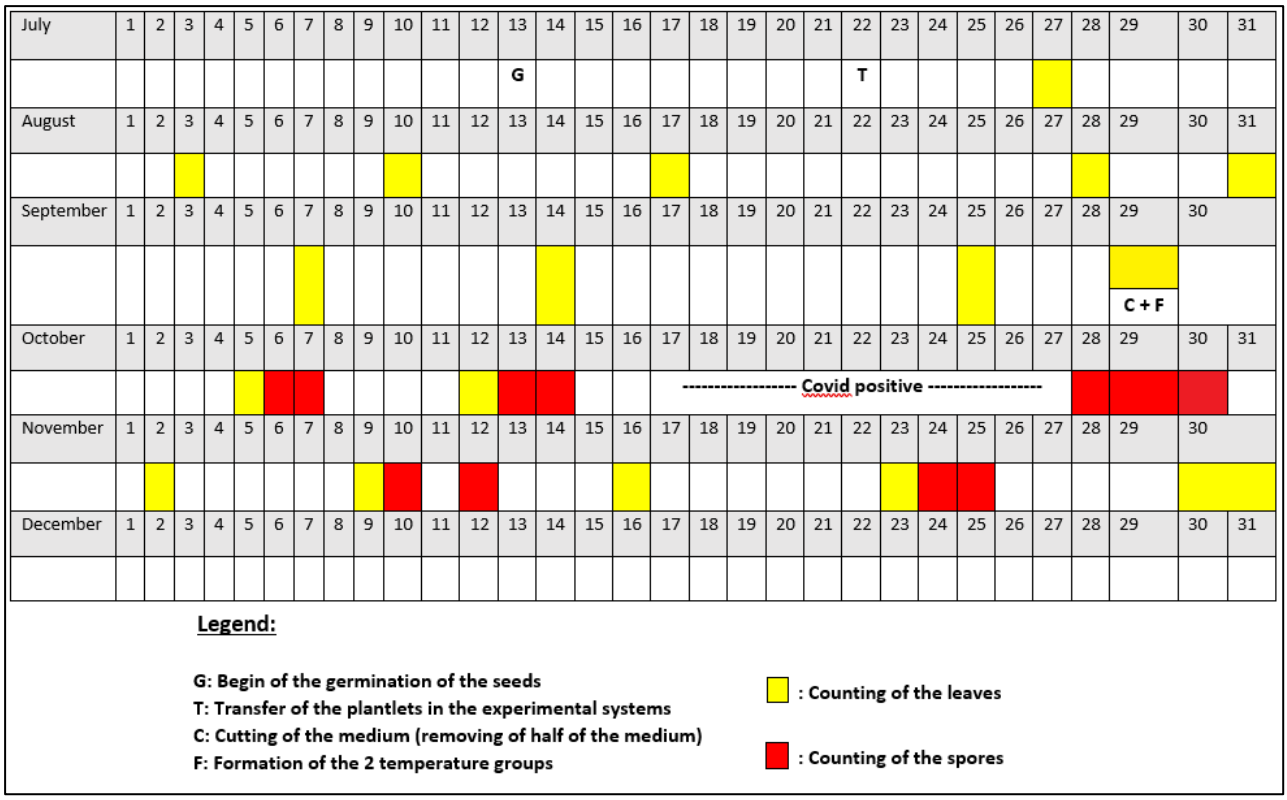


*Figure 21 : This figure shows graphically the calendar that was followed during the experiment. Besides the counting operations, there are feeding operations of the systems under laminar flow hood. These feeding operations are not reported.*

## 13.3 Annexe 3: Detailed graph of the spore production with time for each experimental system

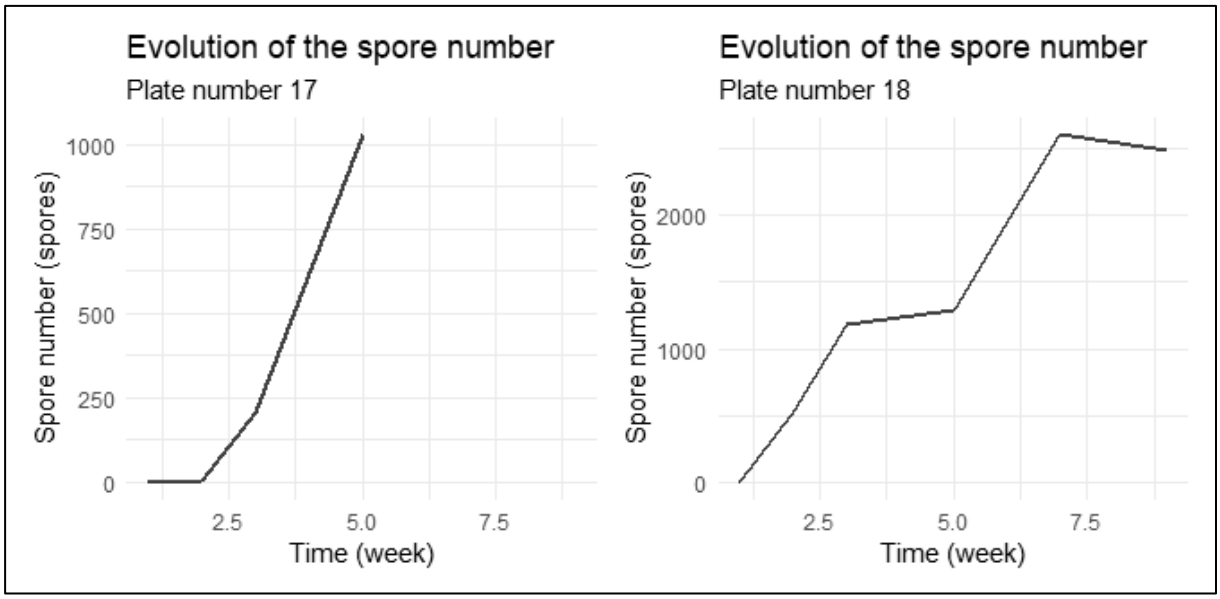


*Figure 22 : Graph of the spore evolution with time in the systems number 17 (R.irregularis, 24°C) and 18 (R.irregularis, 27°C).*

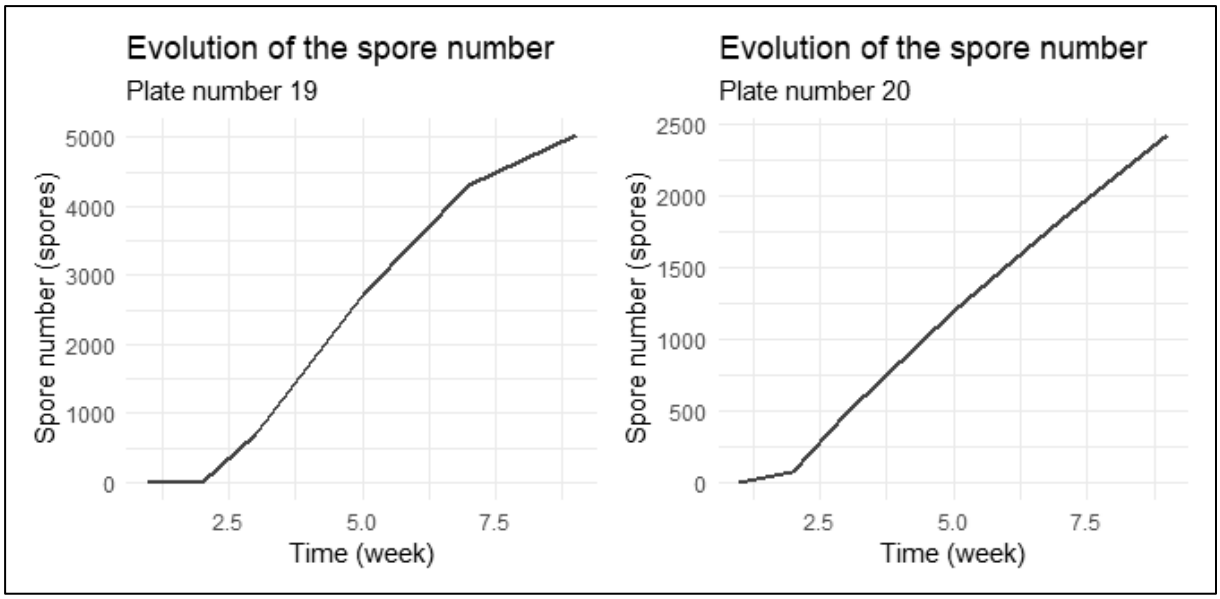


*Figure 23 : Graph of the spore evolution with time in the systems number 19 (R.irregularis, 24°C) and 20 (R.irregularis, 27°C).*

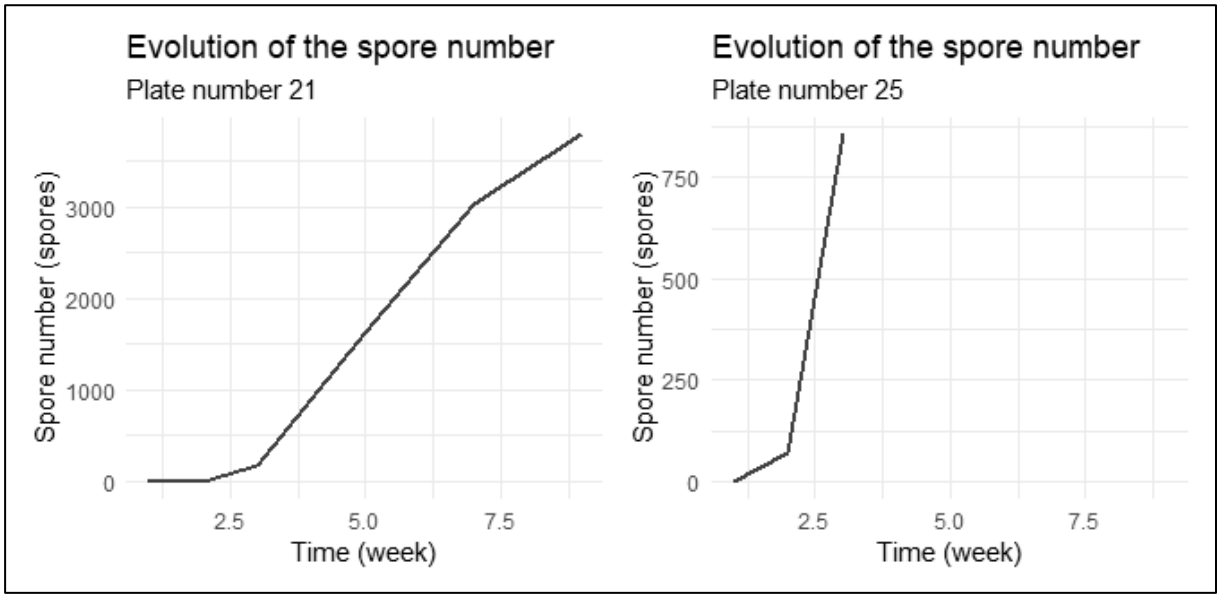


*Figure 24 : Graph of the spore evolution with time in the systems number 21 (R.irregularis, 24°C) and 25 (R.irregularis, 27°C).*

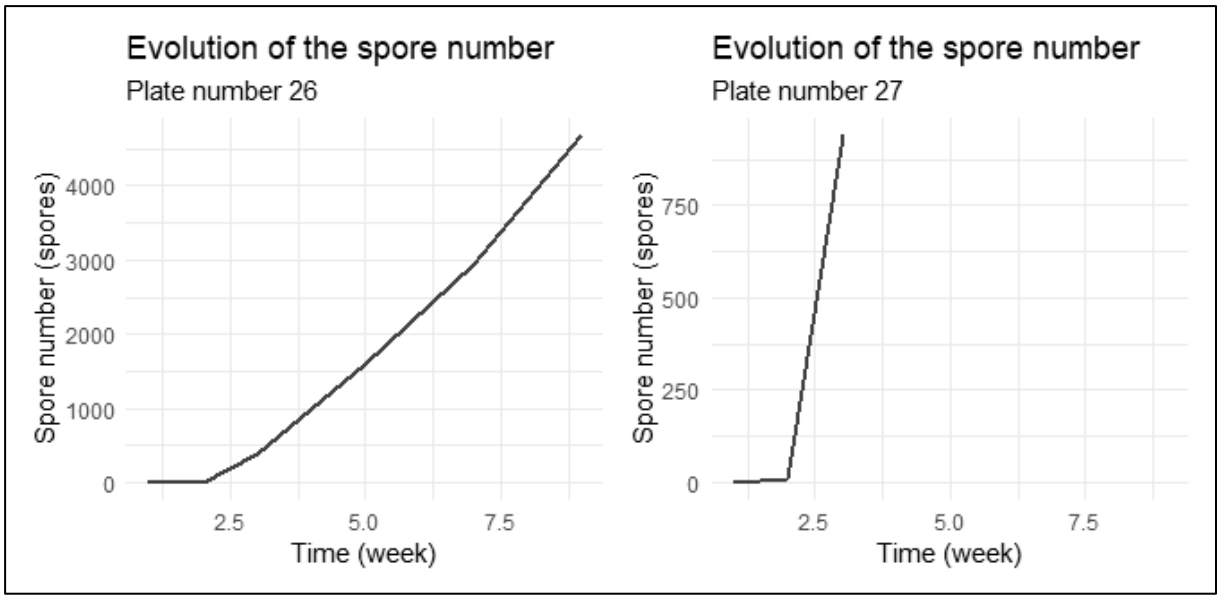


*Figure 25 : Graph of the spore evolution with time in the systems number 26 (R.irregularis, 24°C) and 27 (R.irregularis, 27°C).*

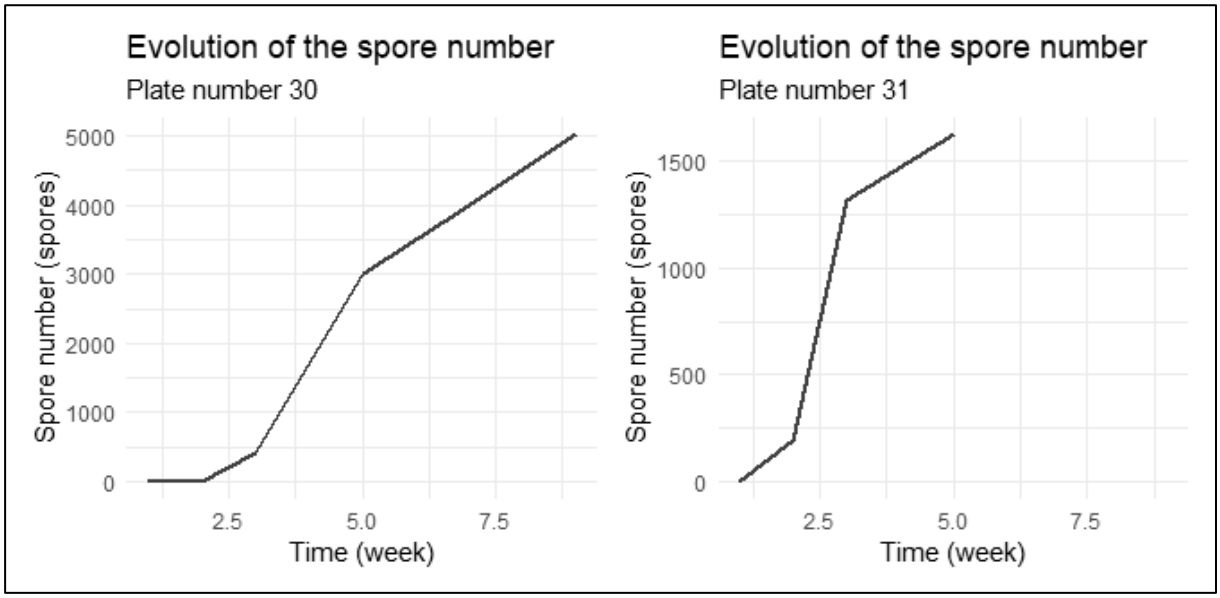


*Figure 26 : Graph of the spore evolution with time in the systems number 30 (R.irregularis, 24°C) and 31 (R.irregularis, 27°C).*

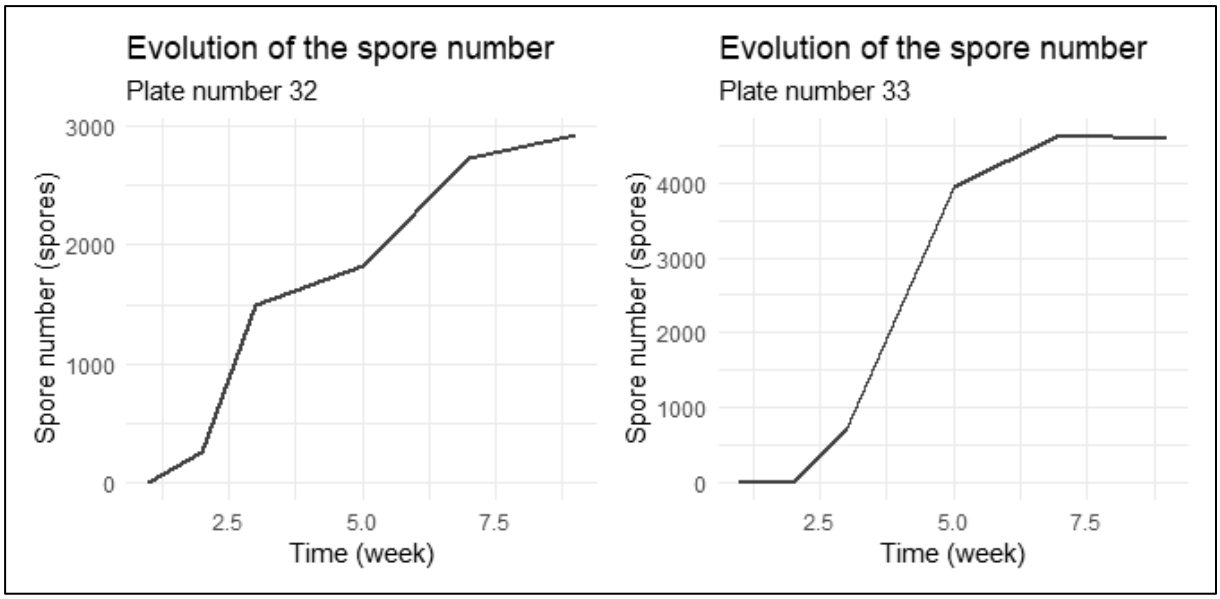


*Figure 27 : Graph of the spore evolution with time in the systems number 32 (R.irregularis, 27°C) and 33 (R.diaphanus, 24°C).*

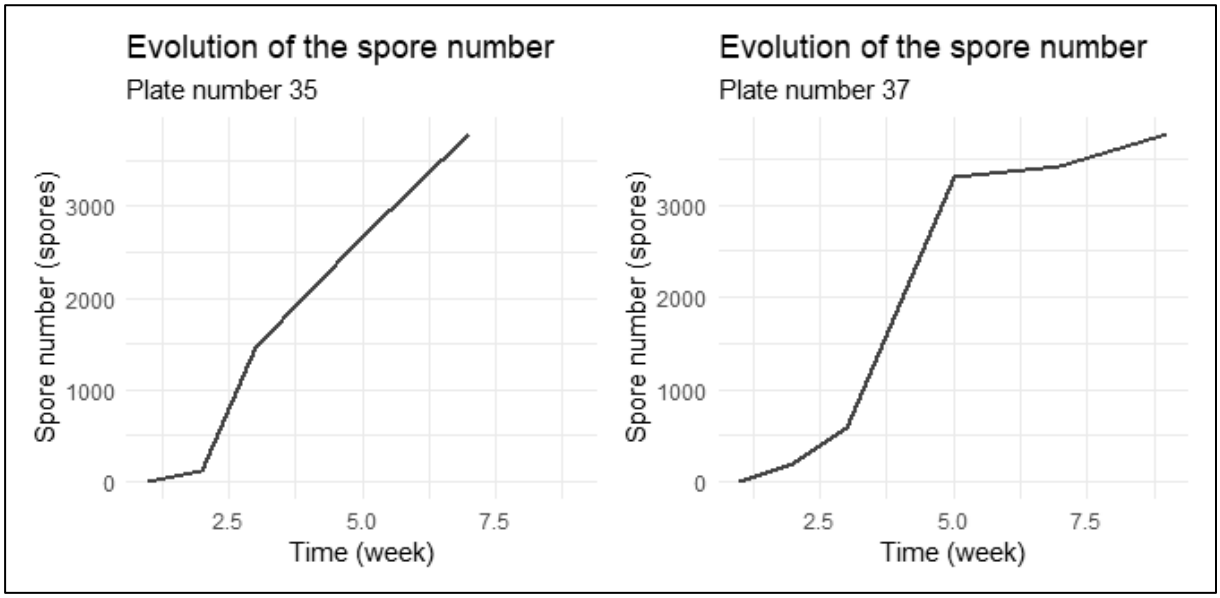


*Figure 28 : Graph of the spore evolution with time in the systems number 35 (R.diaphanus, 27°C) and 37 (R.diaphanus, 27°C).*

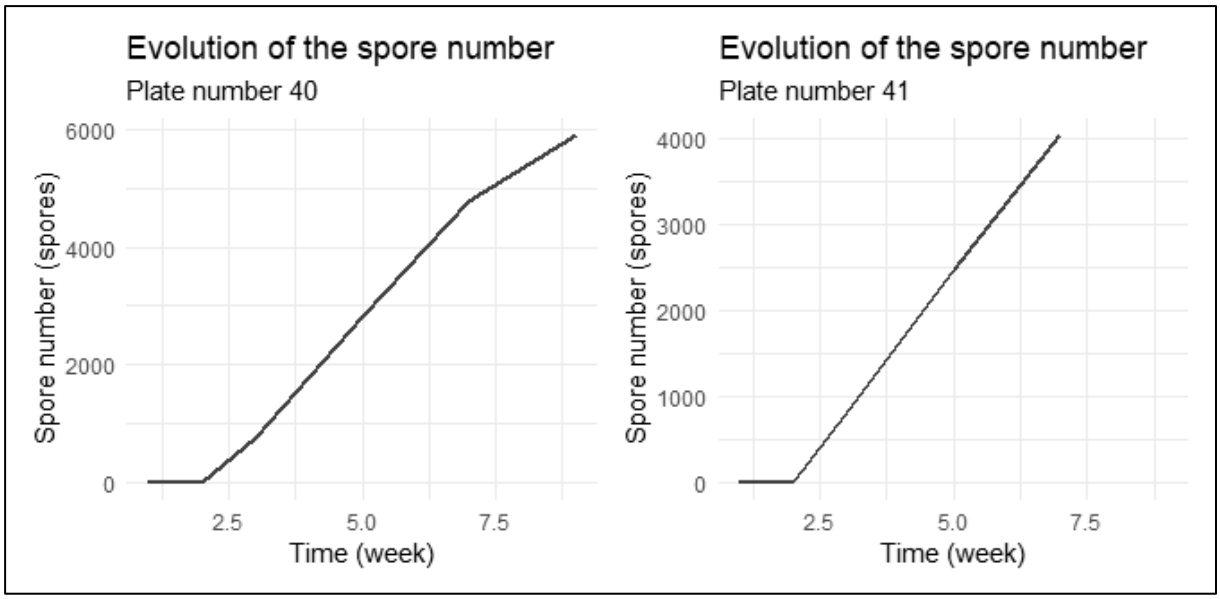


*Figure 29 : Graph of the spore evolution with time in the systems number 40 (R.diaphanus, 24°C) and 41 (R.diaphanus, 24°C).*

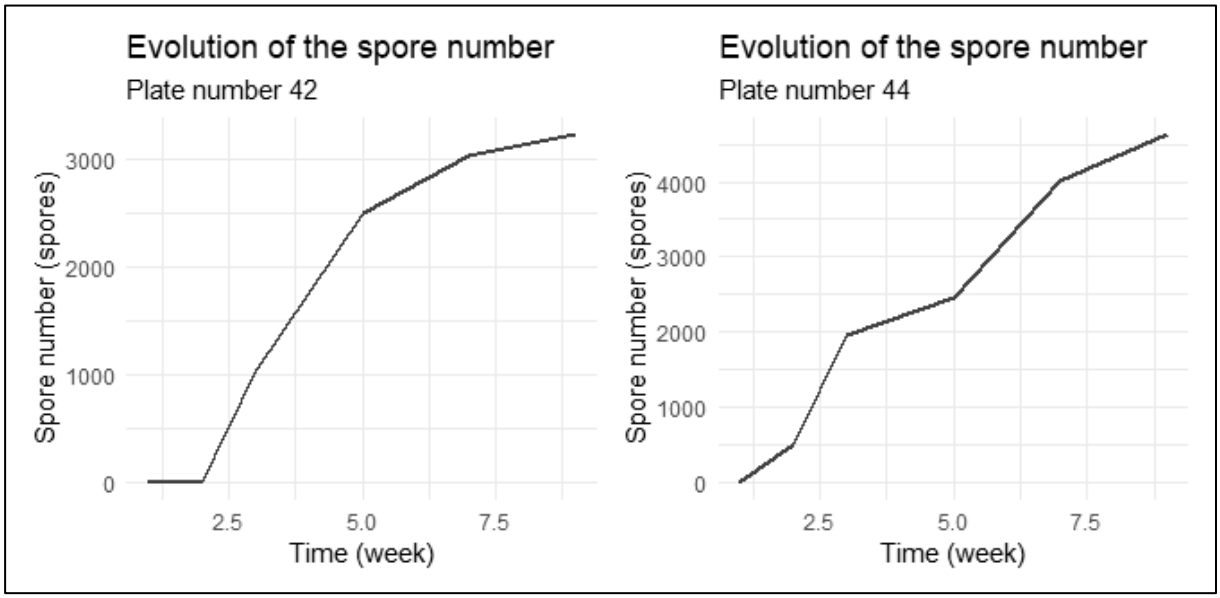


*Figure 30 : Graph of the spore evolution with time in the systems number 42 (R.diaphanus, 24°C) and 44 (R.diaphanus, 27°C).*

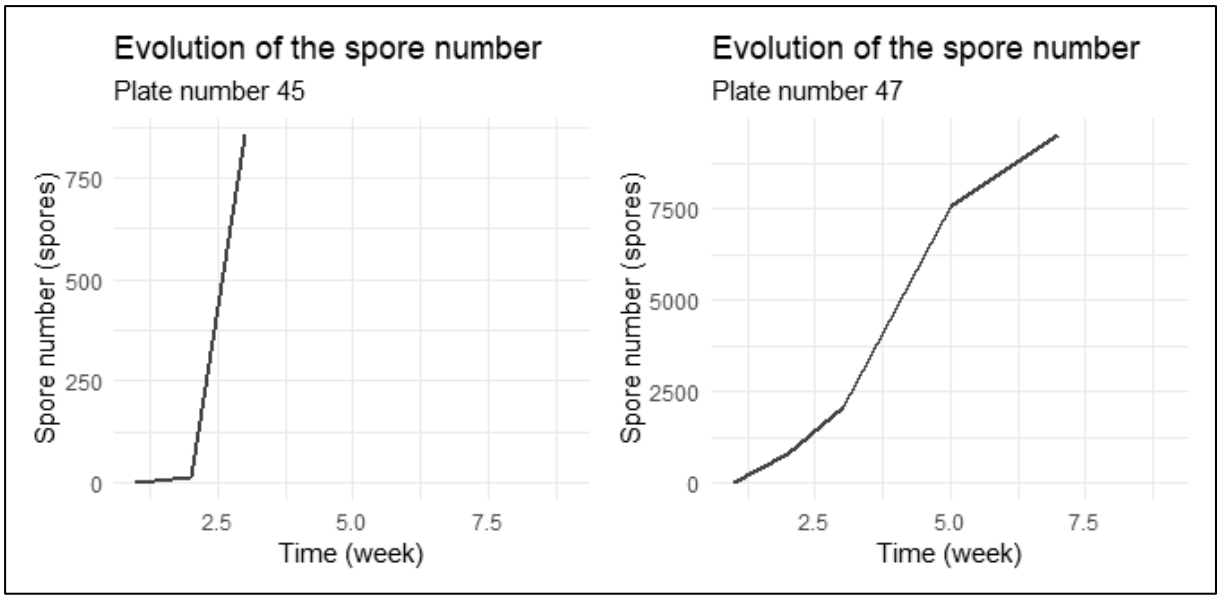


*Figure 31 : Graph of the spore evolution with time in the systems number 45 (R.diaphanus, 27°C) and 47 (R.diaphanus, 27°C).*

## 13.4 Annexe 4: R-output of the model with the lowest AIC and R-output with the coefficients of each adjusted curve in this model

```
> summary(model12)
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: sqrt(Spores) ~ I(Time^2) + Species + Time * Temperature + (Time |      Plate)
   Data: data

REML criterion at convergence: 646.2

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.23108 -0.62073  0.06223  0.52123  2.12586

Random effects:
 Groups   Name        Variance Std.Dev. Corr
 Plate    (Intercept)  0.4333  0.6582
          Time         0.5381  0.7336   -1.00
 Residual             38.5396  6.2080
Number of obs: 99, groups:  Plate, 19

Fixed effects:
                   Estimate Std. Error       df t value Pr(>|t|)
(Intercept)         -22.8837     2.5237  85.7127  -9.067 3.69e-14 ***
I(Time^2)            -1.0164     0.1073  82.7737  -9.471 7.67e-15 ***
SpeciesRi            -3.6577     1.5449  25.9188  -2.368  0.02565 *
Time                 19.3391     1.1172  84.8175  17.310  < 2e-16 ***
TemperatureT27        9.9765     2.3359  64.9110   4.271 6.49e-05 ***
Time:TemperatureT27  -2.1519     0.6200  15.3491  -3.471  0.00332 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) I(T^2) SpecsR Time   TmpT27
I(Time^2)    0.675
SpeciesRi   -0.346 -0.004
Time        -0.809 -0.929  0.007
TempertrT27 -0.486 -0.054 -0.033  0.246
Tm:TmprtT27  0.360  0.055  0.009 -0.297 -0.755
> |
```

*Figure 32 : R output for the model with the lowest AIC. A lot of information is represented. There is for example the formula of the model, the variance of the random effects, the estimate, the standard error and the p-value of the fixed effects, the number of observations and the number of groups (plates).*

```
> coefficients(model12)
$`Plate`
      (Intercept) I(Time^2) SpeciesRi     Time TemperatureT27 Time:TemperatureT27
no_17   -22.17642 -1.016384 -3.657669 18.55082       9.976546            -2.151867
no_18   -22.55977 -1.016384 -3.657669 18.97804       9.976546            -2.151867
no_19   -23.42304 -1.016384 -3.657669 19.94009       9.976546            -2.151867
no_20   -22.09344 -1.016384 -3.657669 18.45835       9.976546            -2.151867
no_21   -22.44388 -1.016384 -3.657669 18.84889       9.976546            -2.151867
no_25   -22.90007 -1.016384 -3.657669 19.35727       9.976546            -2.151867
no_26   -22.73466 -1.016384 -3.657669 19.17294       9.976546            -2.151867
no_27   -22.86960 -1.016384 -3.657669 19.32332       9.976546            -2.151867
no_30   -23.35195 -1.016384 -3.657669 19.86087       9.976546            -2.151867
no_31   -22.95090 -1.016384 -3.657669 19.41392       9.976546            -2.151867
no_32   -22.88499 -1.016384 -3.657669 19.34048       9.976546            -2.151867
no_33   -23.21644 -1.016384 -3.657669 19.70985       9.976546            -2.151867
no_35   -23.23367 -1.016384 -3.657669 19.72905       9.976546            -2.151867
no_37   -23.08943 -1.016384 -3.657669 19.56831       9.976546            -2.151867
no_40   -23.42055 -1.016384 -3.657669 19.93732       9.976546            -2.151867
no_41   -22.91095 -1.016384 -3.657669 19.36941       9.976546            -2.151867
no_42   -22.27551 -1.016384 -3.657669 18.66124       9.976546            -2.151867
no_44   -23.56230 -1.016384 -3.657669 20.09530       9.976546            -2.151867
no_45   -22.69294 -1.016384 -3.657669 19.12645       9.976546            -2.151867

attr(,"class")
[1] "coef.mer"
>
```

*Figure 33 : R-output with the coefficients of the curve adjusted for each plate in the selected model (lowest AIC).*

## 13.5 Annexe 5: R-output of the model testing the presence of an interaction between the temperature and the AMF species

```
> summary(model15)
Linear mixed model fit by REML. t-tests use Satterthwaite's method [
lmerModLmerTest]
Formula:
sqrt(Spores) ~ I(Time^2) + Species + Time * Temperature + Temperature *
    Species + (Time | Plate)
   Data: data

REML criterion at convergence: 642

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.15527 -0.59258  0.02306  0.49455  2.14008

Random effects:
 Groups   Name        Variance Std.Dev. Corr
 Plate    (Intercept)  0.2499  0.4999
          Time         0.5538  0.7442   -1.00
 Residual             38.5412  6.2082
Number of obs: 99, groups:  Plate, 19

Fixed effects:
                        Estimate Std. Error       df t value Pr(>|t|)
(Intercept)              -22.6351     2.6806  88.0319  -8.444 5.70e-13 ***
I(Time^2)                 -1.0173     0.1074  81.2267  -9.475 8.92e-15 ***
SpeciesRi                 -4.0943     2.2546  21.0019  -1.816  0.08368 .
Time                      19.3389     1.1182  83.6971  17.295  < 2e-16 ***
TemperatureT27             9.3503     2.9760  85.4752   3.142  0.00231 **
Time:TemperatureT27       -2.1503     0.6250  15.0948  -3.440  0.00361 **
SpeciesRi:TemperatureT27   1.0642     3.1701  23.3995   0.336  0.74009
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Correlation of Fixed Effects:
            (Intr) I(T^2) SpecsR Time   TmpT27 T:TT27
I(Time^2)    0.635
SpeciesRi   -0.471 -0.001
Time        -0.758 -0.928  0.005
TempertrT27 -0.564 -0.041  0.424  0.190
Tm:TmprtT27  0.336  0.055 -0.008 -0.298 -0.591
SpcsR:TmT27  0.333 -0.002 -0.711 -0.001 -0.622  0.020
convergence code: 0
boundary (singular) fit: see ?isSingular
```

*Figure 34 : R-output of the model taking into account the interaction between the temperature and the AMF species. This interaction is not significant (p-value > 0,05).*

## 13.6 Annexe 6: R-output of the model testing the presence of a counter effect

```
> summary(model6)
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: sqrt(Spores) ~ I(Time^2) + Time * Scientist + (Time | Plate)
   Data: data

REML criterion at convergence: 538.6

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.57945 -0.58738  0.06341  0.49995  1.89346

Random effects:
 Groups   Name        Variance Std.Dev. Corr
 Plate    (Intercept) 68.014   8.247
          Time         3.105   1.762    -0.84
 Residual             32.606   5.710
Number of obs: 80, groups:  Plate, 19

Fixed effects:
                Estimate Std. Error       df t value Pr(>|t|)
(Intercept)     -30.5339     3.8789  56.1887  -7.872 1.23e-10 ***
I(Time^2)        -1.3620     0.1485  47.8833  -9.170 4.08e-12 ***
Time             22.7145     1.6188  53.0445  14.032  < 2e-16 ***
ScientistT       -1.2646     5.5819  60.5738  -0.227    0.822
Time:ScientistT  -0.4728     1.1922  24.5890  -0.397    0.695
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:
            (Intr) I(T^2) Time   ScntsT
I(Time^2)    0.751
Time        -0.907 -0.927
ScientistT   0.070  0.347 -0.201
Tim:ScntstT  0.019 -0.267  0.074 -0.907
>
```

*Figure 35 : R-output of the linear mixed model taking the counter into account. Neither the counter effect nor its interaction with the time are significant (p-value > 0,1).*

## 13.7 Annexe 7: R-outputs of the models testing a leaf effect

```
> summary(model10)
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: sqrt(Spores) ~ Time + I(Time^2) + Leaves * Temperature + (Time |      Plate)
   Data: data

REML criterion at convergence: 653.4

Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.9663 -0.6611  0.1142  0.6621  2.3487

Random effects:
 Groups   Name        Variance Std.Dev. Corr
 Plate    (Intercept)  1.019   1.009
          Time         1.103   1.050    -1.00
 Residual             38.418   6.198
Number of obs: 99, groups:  Plate, 19

Fixed effects:
                     Estimate Std. Error       df t value Pr(>|t|)
(Intercept)          -16.2433    12.1545  92.0924  -1.336   0.1847
Time                  17.6789     1.1525  87.1554  15.340  < 2e-16 ***
I(Time^2)             -0.9064     0.1180  85.0790  -7.683 2.43e-11 ***
Leaves                -0.4588     1.0472  92.7631  -0.438   0.6623
TemperatureT27       -18.4113    14.2623  89.9351  -1.291   0.2000
Leaves:TemperatureT27   2.0212     1.2059  89.7637   1.676   0.0972 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) Time   I(T^2) Leaves TmpT27
Time         0.101
I(Time^2)   -0.061 -0.941
Leaves      -0.983 -0.254  0.198
TempertrT27 -0.875 -0.107  0.000  0.869
Lvs:TmprT27  0.862  0.104  0.007 -0.863 -0.993
convergence code: 0
boundary (singular) fit: see ?isSingular
```

*Figure 36 : R-output of the first model testing a leaf effect. The leaf number and the interaction between the leaf number and the temperature are both non-significant (p-value > 0,05).*

```
> summary(model11)
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: sqrt(Spores) ~ Temperature + I(Time^2) + Leaves * Time + (Time |      Plate)
   Data: data

REML criterion at convergence: 658.6

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.82101 -0.65463  0.02375  0.66240  2.32909

Random effects:
 Groups   Name        Variance Std.Dev. Corr
 Plate    (Intercept)  0.1471  0.3835
          Time         0.7762  0.8810   -1.00
 Residual             39.6122  6.2938
Number of obs: 99, groups:  Plate, 19

Fixed effects:
               Estimate Std. Error       df t value Pr(>|t|)
(Intercept)    -18.7426    13.0177  73.4170  -1.440  0.15418
TemperatureT27   4.8659     1.7533  30.3157   2.775  0.00936 **
I(Time^2)       -0.8726     0.1198  85.1962  -7.283 1.52e-10 ***
Leaves          -0.2131     1.1098  72.1328  -0.192  0.84825
Time            14.7278     2.1849  89.4440   6.741 1.49e-09 ***
Leaves:Time      0.2204     0.1543  92.7336   1.428  0.15655
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) TmpT27 I(T^2) Leaves Time
TempertrT27 -0.315
I(Time^2)    0.059  0.032
Leaves      -0.985  0.252  0.071
Time        -0.746  0.136 -0.609  0.668
Leaves:Time  0.881 -0.180  0.130 -0.881 -0.850
>
```

*Figure 37 : R-output of the second model testing the presence of a leaf effect. The leaf number and its interaction with the time are both non-significant (p-value > 0,1).*

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