Università degli Studi di Padova

Department of Agronomy, Food, Naturals Resources, Animals and Environment

Masters of Science (MSc) in Sustainable Agriculture

Rhizophagus Irregularis Responds Differentially to Commercial Fungicides in* in Vitro *Root Organ Cultures

Supervisors :

Prof. Andrea Squartini

Dr. Daniel Lammel

Master's Student :

Henri Lucien Sandifer

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Abstract

Arbuscular Mycorrhizal Fungi are an ancient asexual type of fungi that undergo symbiosis with a majority of land plants and provide them essential services. Modern agriculture's use of fungicides has the potential to hinder this important symbiosis by damaging these fungi and their capacity to reproduce. While certain studies have looked at the effect of fungicides in the field or in greenhouse experiments, few have looked at the effect of fungicides on AMF in controlled laboratory environments using a new culture method referred to as a root organ culture. This petri dish culture method allows for a far greater control of variables affecting the growth of the fungus as well as greater visibility of the fungus due to the optical transparency of the growth medium. The commercial fungicides used in this experiment demonstrated an effect on AMF growth, in some cases decreasing biomass, increasing spore count and producing abnormal growth phenotypes. The detrimental effect of fungicides on AMF ought to be well documented as they are fundamental in soil and plant health both in natural and agroecosystems.

1. Introduction

With the advent of modern agriculture, increasing pressure has been placed on agroecosystems to withstand the threat of pathogens, especially fungal pathogens. Monocultures favor the proliferation of pathogenic fungi (Shipton, 1977), whose development in the field and post-harvest can lead to disastrous food loss. A number of pathogen management strategies have emerged in order to prevent and limit the damage caused to crops by fungal pathogens in conventional production systems, the most significant of which have been synthetic fungicides, which are able to target essential biochemical pathways in pathogenic fungi so as to limit their spread and impact.

The use of conventional pesticides, however, does not come without its share of issues. While the constraints placed on fungicides are now far more stringent than they were when this class of compounds first started seeing wide scale application in the 1970s, fungicides still disperse and concentrate in terrestrial and aquatic ecosystems, harming non-target organisms and in certain cases making their way into the human body (Yang et al., 2011; Zubrod et al., 2019).

One of the said non-target organisms which fungicides can harm, and whose damage may have a directly negative effect on the crops the fungicides aim to protect, are the class of beneficial fungi known as mycorrhizal fungi. These soil-dwelling fungi are of great importance to terrestrial ecosystems (Zhang et al., 2022) and the plants within them, and a poorly managed use of fungicides could present significant risks for them. Few studies have looked at the effect of commercial fungicides on these fungi, especially in *in vitro* systems in which direct, non-confounded effects can be observed.

2. Mycorrhizal fungi

Mycorrhizal symbiosis is a widespread ecological phenomenon occurring with 80-90% of land plants (Smith & Read, 2010). Among organisms partaking in this commensal relationship, the most common plant-symbiotic fungi are Arbuscular Mycorrhizal Fungi (AMF) (Avio et al., 2018). AMF are typically non-specific, meaning they can establish symbiosis with hosts from various species (Selosse et al., 2006). They are an ancient asexual lineage of fungi belonging to the monophyletic phylum *Glomeromycota*, which is believed to have emerged around 460 million years ago and to have played a pivotal role in the evolution of terrestrial plants - whose emergence also dates back to the same time period (Schüβler et al., 2001), (Remy et al., 1994).

Mycorrhizal fungi are obligate symbionts, meaning their life cycle depends on an ongoing symbiosis with one or more plants. As opposed to the other type of mycorrizae, Ectomycorrhizal fungi, whose entire lifecycle takes place outside the host plant's roots, Arbuscular Mycorrhiza penetrate their host's roots, colonizing the root cortex cells in which they form a branched structure within individual cells known as an arbuscule, the locus of exchange between the fungus and the plant (Delian et al., 2017). The main resource for which plants typically recruit mycorrhizal fungi is phosphorus (Smith & Read 2010). When plants detect a lack of phosphorus in the soil, they will produce compounds known as "strigolactones" whose concentration gradient in the soil will guide the fungi towards the plant roots through a process of chemotaxis (Besserer et al., 2006).

AMF have long been known to provide their host plants and the soil with a number of benefits (Bethlenfalvay, 1992). Such services include the provision of water and minerals (Bowles et al., 2016)**,** immunity enhancement (Mathur & Vyas, 1996), (Lowe et al., 2012), protection against a number of pathogens such as, for instance, *Rhizoctonia solani* (Iqbal & Mahmood, 1986),

Phytophtora infestans (Gallou et al., 2011) or *Botryosphaeria* (Krishna et al., 2010), relief from toxic levels of salinity (Wang et al., 2023), metals (Riaz et al., 2021) (Delian, 2011) (Tedersoo et al., 2020) and other sources of abiotic stress such as heat, drought and extreme temperatures (Begum et al., 2019). AMF will not merely establish symbiosis with a single host but will connect multiple plants to one another (referred to as a Common Mycorrhizal Network) through which resources such as photosynthates are transferred between plants (Tedersoo et al., 2020). A meta-analysis of observations from 1950-2021 looking at the effect of AMF inoculation on the yield of various crops under rainfed conditions showed an overall yield increase of 23% due to improvements in plant nutrition, photosynthesis efficiency and stress resistance (Wu et al., 2022). AMF inoculation has also been shown to improve a number of agronomic factors such fiber quality in cotton (Gao et al., 2020), essential oil concentration in basil (Yilmaz & Karik, 2022), or bioactive compounds and antioxidants in saffron (Caser et al., 2019). In exchange, it is estimated that plants will provide up to 10-20% of their carbon fixed via the calvin cycle to mycorrhizal fungi (Řezáčová et al., 2017).

In addition to connecting plants to one another and providing them with valuable services, AMF play a crucial role in the health of the soil itself, indirectly through their interactions with plants and soil microorganisms that shape the soil ecosystem but also through direct soil engineering (M. Gupta, 2020).

As far as agriculture is concerned, then, mycorrhizal fungi play two key roles in rendering the practice sustainable : plant production and soil quality. A number of conventional agricultural practices, however, are known to be detrimental for mycorrhizal fungi such as high phosphorus fertilization (Konvalinková et al., 2017), soil tillage and the application of fungicides used to manage the proliferation of pathogenic fungi (Trappe et al., 1984) (Pfleger & Linderman, 1994). Fungicides play an undeniable role in guaranteeing stable agriculture production cycles, but they are also an obvious threat to AMF. As long as they are part of disease management strategies, direct and indirect costs will be incurred not only by the surrounding ecosystem, but also by the given crop, whose yield and quality may be hindered by the damage done to the potentially critical symbiotic organisms that are AMF. The threat which pathogenic fungi place on agricultural production must thus be tackled by

as many effective angles as possible to reduce the harm they cause without leading to excessive collateral environmental and agronomic damage as a result of their application.

3. Fungal pathogens

While some fungi, such as AMF, stand as direct allies of agricultural production, other members of the fungal kingdom prove to be the most significant pathogen affecting agriculture and forestry, causing over 70% of diseases affecting agricultural plants (Oerke, 2006). Fungal pathogens cause up to 20% of yield losses globally, with an additional 10% loss of food goods post-harvest (Fisher et al., 2018), jeopardizing yields, food quality and safety. Worldwide, annual crop losses due to plant diseases as a whole are estimated to cost up to $220 billion (FAO - News Article, n.d.)). Pathogenic fungi use microbe-associated molecular pattern (MAMP) molecules as a part of five main strategies to overcome their plant host defenses and colonize when they 1) attach to their host, 2) avoid or suppress its detection systems, 3) colonize its intercellular space, 4) modify its cell structure and functions, and 5) facilitate their own growth and reproduction (Tör & Woods-Tör, 2017). So as not to leave the crops on which human society depends at the mercy of pathogens, agronomists have opted to go head-to-head with pathogenic fungi, traditionally using substances such as copper sulfate to combat them, and since the chemical and agronomic revolution of the mid twentieth century, relying far more heavily on synthetic fungicides.

4. Chemical Control of Pathogenic Fungi

Synthetic fungicides that inhibit or kill pathogenic fungi have seen a vast increase in use around the globe since the 1950s to limit the damage which these pathogens inflict on agricultural production (Russell, 2006). With the rapid increase in human population size and consequent increase

in the demand for food that has occurred over the past seventy years, in 2009 pesticide use worldwide was estimated at 2 million tons, 45% of which were in Europe and 24% in the USA (Abhilash & Singh, 2009). The EU pesticide database states that 147 of 496 approved active substances belong to the category of fungicides (*Approval of Active Substances*, n.d.), despite accounting for 46% of all pesticides sold in the EU. The most widespread classes of antifungal compounds used in fields include benzimidazoles, dithiocarbamates, strobilurins, and azoles (Hof, 2001).

Despite efforts to curb this trend and adopt integrated and comprehensive pest management strategies, reliance on fungicides in agriculture is increasing (Tleuova et al., 2020). To add to the ease with which fungal pathogens tend to adapt to conventional agricultural systems, particularly monocultures (Stukenbrock & McDonald, 2008), in the coming years and decades climate change and fungal migration patterns may also contribute to the pressure which fungal pathogens place on agricultural production (Garrett et al., 2021). Pathogenic fungi have a number of evolutionary strategies at their disposal which contribute to their expansion worldwide, such as an ease of infection and host-plant species transfer, life cycle adaptation, and a variety of propagation methods occurring via long-distance, airborne spore dispersal, in great part due to human activity (Naranjo-Ortiz & Gabaldón, 2019).

While effective in controlling the proliferation and potential harm caused by the pathogens, fungicides pose a set of risks that has historically garnered less attention than other pesticides such as insecticides and herbicides, and which may be of concern to the crops themselves ((Zubrod et al., 2019). When fungicide application protocols are not managed properly, they place a high selective pressure on the target pathogen, effectively assisting the proliferation of resistant strains. Documented cases of fungicides placed on the market that have become ineffective against their target pathogens date all the way back to 1960, many of which have become ineffective due to pathogen resistance in only two years (Lucas et al., 2015). To date, there are four main mechanisms behind the development of fungicide resistance: (i) mutation induced target protein alterations (ii) upregulation of target proteins, (iii) increase in efflux processes (iv) degradation of the fungicide due to detoxification by metabolic enzymes (Sanglard, 2016) (Scorzoni et al., 2017) (Lucas et al., 2015).

As stated above, fungicides have been observed to negatively affect a number of non-target organisms in both terrestrial and aquatic mechanisms (Zubrod et al., 2019) and up until recently, their exact mode of actions had only been poorly classified and the extent of their environmental contamination underestimated (Yang et al., 2011). Their impact on human health is also not to be overlooked, as a number of them have been shown to present health risks for humans as carcinogens, endocrine disruptors and presenting reproductive and developmental toxicity (Karabelas et al., 2009) (Munger et al., 1997). Among the non-target organisms they affect are AMF, whose loss or hindrance due to fungicide applications has the potential to directly and negatively impact crop production.

While fungicides offer a relatively reliable solution to fungal pest management, they are not the only tools in our agronomic arsenal. In fact, as mentioned previously, AMF too help their host plants avoid or respond to disease, through direct underground competition in the case of soil-borne pathogens or through immune response enhancement. While AMF may not be sufficient alone to combat current pathogens, they may constitute collateral damage in the battle against pathogenic fungi that we cannot sustainably afford.

5. Effects of Fungicides on AMF

A number of studies dating back to the 1960s have documented the effect of fungicides on AMF, but the work is far from over, as the constant release of new fungicidal compounds on the market make the study of these compounds on AMF a constantly renewed labor. Evidently, given the variety of modes of actions within the arsenal of fungicidal compounds used in agriculture, modes of action which are often poorly known, characteristics of the soil as well as the sheer diversity of AMF species present in agricultural soils and the host plants in question, the effect on AMF will vary in type and severity. To add to the complexity of studying the matter, it is not always clear at what concentrations fungicides are present in any given location in the soil, and thus estimating to what degree they will impact AMF populations is challenging. So far, however, the vast majority of studies

demonstrated a negative impact of fungicides on AMF for a number of parameters, with few studies revealing neutral or positive effects on the plant symbionts.

Fungicides typically fall into two main categories : contact and systemic. Contact fungicides, on the one hand, do not penetrate plant tissues but merely reside on their surface and prevent the initial steps of fungal infection. Systemic fungicides, on the other hand, will penetrate into the plant and circulate within it via the plant phloem - though to varying degrees, with some systemic compounds remaining localized - for both preventative and curative action against fungal infections.

At a first glance, systemic fungicides pose an evidently greater risk of coming into contact with AMF as they circulate inside the plant and thus gain direct access to the cells in which AMF arbuscules are present. Contact fungicides will only affect AMF by making their way to the AMF externally through infiltration into the soil after pulverization in the field. That being said, this second class can still prove quite destructive - a greenhouse study looking at contact fungicides Octagon, Ditiver, Octagon, Parmex observed a complete elimination of symbiosis (Hernández-Dorrego & Mestre-Parés, 2010). Since systemic fungicides dominate the market, however, they remain the focus of most studies.

Studies looking at the effect of fungicides on AMF tend to look at the effect of fungicides either in their commercial formulations, including surfactants, emulsifiers, and so on, or at the active fungicidal ingredients alone, typically accompanied by a solvent such as ethanol, DMSO or acetone - which, when used alone as control treatments, tend not to harm AMF. Studies cited below will indicate commercial formulations with capitalization and active ingredients in parentheses. Parameters that are typically measured in these studies are spore production and germination, root colonization, extraradical mycelium development, mycelium architecture and germ tube elongation. Open field and greenhouse tests have been carried out since the 1970s to test these effects, while *in vitro* studies, typically using split-plate root organ cultures, have only emerged since 2008 with Dalpé & Séguin's "paper bridge" method developed in 2010**.**

The ability for AMF to successfully and amply colonize host roots is a core determinant of the degree to which the symbionts can assist their host plants. Anything hindering this core part of the symbiosis inherently limits its benefits to the plant. In open field and greenhouse studies, highly detrimental effects on root colonization have been observed on leek plants for fungicides Rubigan, Frupica, Sinthane, Octagon, Ditiver, Octagon, Parmex (Hernandez-Dorrego et al., 2010), and in pea and chickpea plants for fungicides AllegianceTM FL, Apron Maxx® RTA® , Vitaflo® 280, Crown® and Trilex® (Jin et al., 2013). More neutral effects have also been observed on leek plants with fungicides Beltanol, Previcur, Aliette, Forum, Teldor, Switch, Ortiva (Hernandez-Dorrego et al., 2010) and Agrox® FL and Thiram (Jin et al., 2013).

The consequence for the host plant due to a loss of symbiosis can be notable, and studies have observed an impact of fungicides on a number of indicators of plant health and production mediated by their negative effect on AMF. Studies looking at the dry weight of roots of *Medicago truncatula* exposed to (fenhexamid) and (fenpropimorph) (Zocco et al., 2008), carrot exposed to (fenhexamid) and (fenpropimorph) (Zocco et al., 2011) and cucumber exposed to Benlate, Corbel, Tilt 250EC and Tilt Top (Kjøller & Rosendahl, 2000) observed a significant decrease in biomass as a result of inhibited AMF growth. The opposite has also been observed in a greenhouse study, however, with increases in dry weight and colonization rate, hypothetically due an increase in sugar exudates from roots of VAM-infected plants following fungicide application (Dodd & Jeffries, 1989).

Other indicators of plant development that have been studied focus on various elements of host and AMF biochemistry. Two important enzymes have been studied in this regard that pertain to phosphorus acquisition and transport. These are alkaline phosphatase (ALP) and succinate dehydrogenase (SDH). Alkaline phosphatase acts on organic compounds by cleaving mono-phosphate groups and is often used in soil ecology to indicate microbial activity. A causal connection has been suggested between it and the proportion of hyphae involved in phosphorus metabolism during AM symbiosis (Tisserant et al., 1993). Succinate dehydrogenase (SDH) is an enzyme complex present in many bacterial cells and in the mitochondria of eukaryotes and is the only enzyme which participates in both the citric acid cycle and the electron transport chain (Trumpower, 1990). Due to phosphorus'

vital importance for the host and as the nutrient at the origin of AMF recruitment, the hindrance of its acquisition and transport by the AMF due to the application of fungicides is also likely to be detrimental to the plant.

Fungicides such as Benomyl (Kjøller & Rosendahl, 2000), and (fenpropimorph) and (fenhexamid) (Zocco et al., 2011) have been shown to negatively impact ALP and SDH respectively. Fungicide Benomyl has also been shown to affect malate dehydrogenase (MDH) when applied at the recommended field rate (Thingstrup & Rosendahl, 1994). In another study, however, Jin et al., 2013 observed no noticeable effects on P uptake by AMF host pulse crops pea and chickpea when exposed to Agrox® FL and Thiram 75WP.

As evidenced by these studies, while a trend is present for a negative effect of fungicides on AMF and its services to its host, depending on the crop, fungicide and dose, these effects can vary. Studies in soil substrates are crucial for this as they most closely emulate the real conditions in which plant-AMF symbiosis occurs. As far as study accuracy is concerned, however, soil-based studies make measuring a number of these parameters and isolating compounding factors challenging.

Studying extraradical mycelium, for instance, is particularly difficult in soil substrates since the mycelium cannot be visualized and its fragility makes it easily destroyed during harvest and processing. Only recently have effective *in vitro* methods developed, referred to as monoxenic or root plates organic cultures, which allow for a far more controlled study of the AMF symbiosis. Discrepancies occur in results, nonetheless, between *in vit*ro studies and greenhouse or field studies - some studies reveal different results for parameters such as root colonization between both approaches (Rejali et al., 2022).

The extraradical part of mycelium is of crucial importance to the viability of the symbiosis, as this is the part of the mycelium which acquires resources for the plant. *In vitro* studies examining the effect of fungicides on this part of AMF mycelium virtually all register a negative effect, such as with fungicides Amistar (Buysens et al., 2014), Benomyl, Corbel, Tilt Top (Kjøller & Rosendahl, 2000), and (fenpropimorph) and (fenhexamid) (Zocco et al., 2008).

The final parameter typically studied when looking at fungicidal activity on AMF, which may not have a direct impact on crop production but which may determine the degree to which AMF can subsist in the soil over generations, is the effect of fungicides on spore production and germination. Many fungicides have been found to negatively affect spore germination, such as Bavistin (Dodd et al., 1989) and (fenhexamid) and (fenpropimorph) (Zocco et al., 2008), although this is not the case for all fungicides, as some, such as Amistar (azoxystrobin), Monarch (flutalonil), Monceren (pencycuron) (Buysens et al., 2014) have not been found to be detrimental to this process.

The evolution of plant diseases affecting agriculture systems is not set to stop or slow down in the near future and fungicides appear to be a necessary response to that fact. Hence due to the sheer number of fungicides on the market and their differing (and sometimes poorly understood) modes of action, and the fact that some prove highly detrimental to a number of parameters pertaining to AMF symbiosis while other may be AMF-compatible; and since differing protocols for either modality have led to contradicting results in this field of study, continued studies and the refining of protocols seem essential. Down the line, it will be important to then take regulatory steps to avoid having our crop protection methods backfire, not only harming humans and the ecosystems around us, but also harming the crops they are designed to protect by harming their essential symbionts.

6. Objective of the study

The purpose of this study was to look at the response of AMF to four commercial fungicides that have not yet been studied in *in vit*ro systems. The split-plate modality provided us the capacity to look at the extraradical mycelium, which is near impossible in soil studies. Here we sought to measure the biomass of the extraradical mycelium after exposure to fungicides, conduct a spore count and analyze the extraradical mycelium's architecture. We expected to see a lower biomass than control at the high dose (higher than field rate) for the four fungicides, with variable results at low dose (a fraction of the field rate). As far as spore production and extraradical morphology is concerned, little

is known about factors affecting them and their properties - here the study aimed to provide additional data as a support for further studies.

The fungicides were chosen in their commercial formulations instead of the active ingredients. While studying the latter helps examine how the given molecule selected for its anti-fungal properties will act on the AMF, given that the *in vitro* study is already removed from the complexities of soil conditions, we considered it more relevant to examine the formulations as a whole, including their surfactants and emulsifiers.

7. Materials & methods

7.1. Split Plate Root Organ Cultures

Split-plate root organ cultures are a soil-free method used for the *in vitro* study of AMF. By transforming plant roots with *Agrobacterium tumefaciens*, it is possible to grow plant roots in a petri dish while avoiding the production of shoots or foliage (Willmitzer et al., 1982) - the sugars which the plant would typically derive from photosynthesis and respiration are supplied by the medium and bacteria. In these systems, roots and mycelium are grown in a nutritional medium known as M-medium which contains vitamins and minerals, a stabilized pH between 5.49 and 5.51 and solidified with phytagel.

The modification of a typical root organ culture into a split-plate system restricts the roots to one of the two compartments of the plate, allowing for a proliferation of the AMF mycelium alone in the second compartment. An added benefit of this method compared to soil cultures is the optical transparency of M-medium, allowing for high resolution imagery of the culture as well as an easy harvest and processing of mycelium, spores and colonized roots. These systems have also been used for new investigations into poorly understood phenomena such as the interactions between AMF and

phosphate solubilizing bacteria (Jiang et al., 2021), or the differences in life history traits between AMF homokaryons and dikaryons (Serghi et al., 2021).

7.2. Fungicides Used

This study tested the response of AMF species *Rhizophagus irregularis* to four different systemic commercial fungicides, each at two different concentrations (high and low). The fungicides studied here were Collis, Merpan, Signum and Switch.

Collis

Collis is used to fight powdery mildew on a variety of crops and contains the active ingredients (boscalid) (200g/l) and (kresoxim-methyl) (100g/l). Boscalid is a succinate dehydrogenase (SDH) inhibitor. Kresoxim-methyl is a mitochondrial cytochrome-bc1 complex inhibitor, used against scab on certain fruits and against other fungal diseases. Cytochrome bc1 complexes are the most widespread electron transfer complex involved in energy transduction (Trumpower, 1990).

Switch

Switch is used to treat gray rot and other fungal diseases, such as *Fusarium, Rhizoctonia, Alternavia* and *Botrytis cinerea*. It contains (fludioxonil) (25%) and (ciprodinyl) (37,5%) . Fludioxonil is a contact fungicide that inhibits the transport-related phosphorylation of glucose, limiting mycelium growth rate. The mode of action of fludioxonil is attributed to a hyperactivation of the high osmolarity glycerol (HOG) signaling pathway, though it is not yet clear how osmotic stress is detected or fungicidal activity is initiated within this pathway (Bersching & Jacob, 2021). Cyprodinyl is an anilinopyrimidine used against *Botrytis cinerea*, *P. herpo trichoides* and *M. oryzae*. The biological mode of action of Pyrimidines involves inhibiting methionine biosynthesis and the secretion of hydrolytic enzymes that target plant cell walls. (Waechter et al., 2010). Methionine is important in fungi pathogenicity - assays have demonstrated that MET6 null mutants are benign on both barley and

rice leaves as they are defective in appressorium-mediated penetration and invasive infectious growth. (Hassan et al., 2019)

Signum

Signum contains the active ingredients (boscalid) (267g/kg) and (pyraclostrobin) (67g/kg). Pyraclostrobin is a strobilurin fungicide, which inhibits mitochondrial complex III of fungal and mammalian cells, inducing an accumulation of triglyceride in certain cells (Xiong et al., 2020).

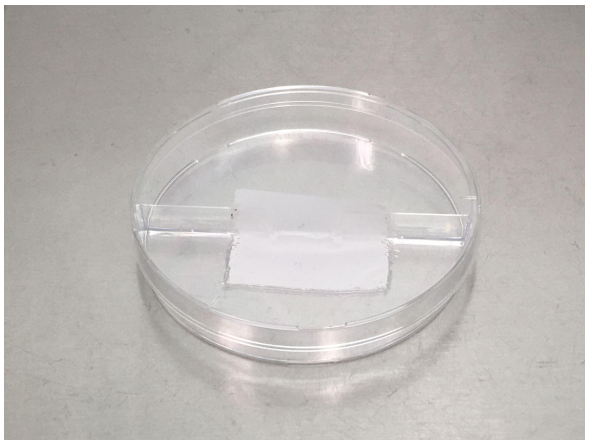
Merpan

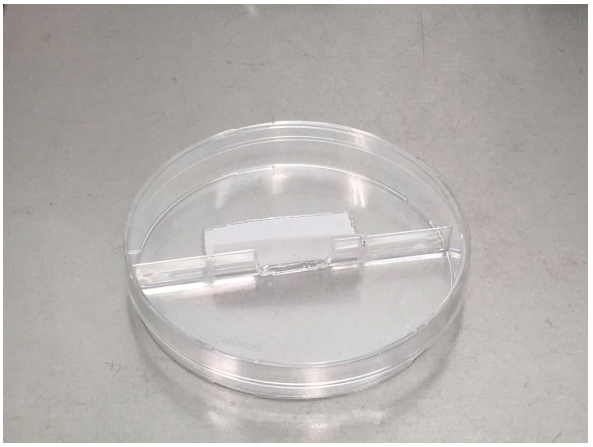
The active ingredient in Merpan is Captan, used to control botrytis, Fusarium, Fusicoccum and Pythium. Captan has a protective and curative action that functions by inhibiting respiration in a number of fungi and bacteria. The fungicidal mode of action is believed to involve degradation components of captan that are highly reactive with thiols and other fungal functional groups (Gordon, 2010).

The doses used for each fungicide used were chosen according to the recommended field-rate application of the compounds, scaled down a petri dish volume based on the quantities of other fungicides used in split plate experiments compared to their respective recommended field applications. The maximum field rate applications for Collis and Switch are 1000g/ha while that of Switch and Signum is 1500g/ha. This equates to 100mg and 150mg of product per m2 of soil, respectively, which in turn equates to 833.3µg/kg and 1250µg/kg of soil respectively. Per volume, this was estimated to be equal to 25µg and 37.5µg of product per 30ml of M-medium respectively. For our high dose, we tripled this quantity, adding up to 75µg and 112.5µg respectively, and for our low dose we divided it by ten, amounting to 2.5µg and 3.75µg respectively.

7.3. Modified Split-Plate System

In this study, an alteration was made to the original "paper bridge" split-plate system designed by Dalpé & Séguin in 2010. While this initial system allowed the mycelium to cross over the barrier dividing both compartments of a split plate, here, a system was developed that would allow for direct passage of the mycelium from the first compartment to the second (**Images 1** and **2**). To this end, a ~2cm section of the dividing barrier was melted away using a soldering iron, and a 30 micron mesh was soldered over the gap, allowing for passage of the mycelium into the second compartment without the mycelium exiting the phytagel medium and without the roots being able to grow through the mesh into the mycelium compartment.





Images 1 (left) and **2** (right) exemplify the split plate, with the central part of the dividing barrier removed and a piece of nylon mesh soldered to the sides of the barrier and to the bottom of the plate (on the "root compartment" side of the plate). The mesh was autoclaved before use and particular attention was given to sterile technique to limit any potential contamination during the plate modification procedure.

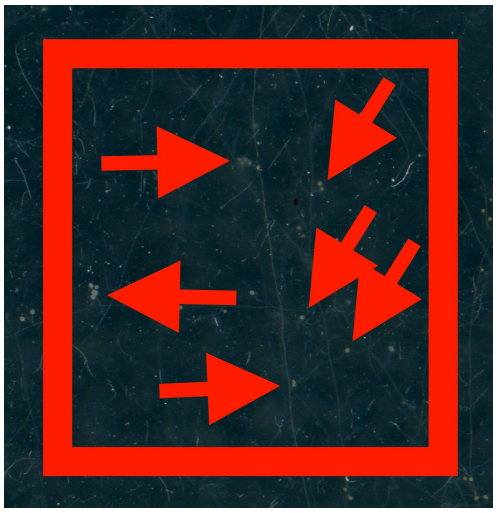
Once the plates were modified, M-medium was prepared according to protocol, autoclaved and maintained at a temperature of 70C° for a maximum of four hours until the experiment was ready to be carried out. Liquid M-medium was poured into the root compartment of the plate and allowed to gel. Colonized roots were obtained from cultures of *R. irregularis* present in the Rillig laboratory's collection at the Freie Universität, Berlin. Sections of cultures containing M-medium and

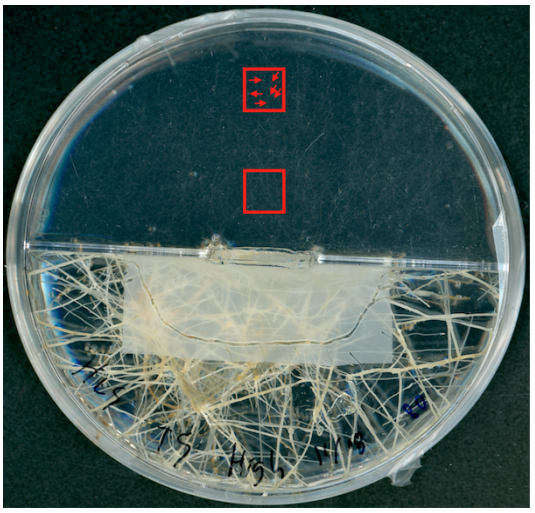
AMF-colonized *daucus carota* roots of equal size and containing similar levels of root growth and extraradical mycorrhizal growth were transferred into the root compartment of the new plates.

For the mycelium compartment where the treatment was to be placed, for each plate, 28ml of M-medium was poured into an erlenmeyer flask, into which the dissolved fungicide was pipetted. Dilutions of each fungicide were prepared using a serial dilution, and 2ml of the final dilution, loaded with either the high or low dose of the given fungicide, was added to the erlenmeyer flask containing the M-medium. The contents of the erlenmeyer flask were mixed manually by swirling the flask clockwise and counter-clockwise for 10 seconds and poured into the mycelium compartment of the split plate. The gel was allowed to cool for approximately ten minutes before placing the lid and sealing the plate, a wait time necessary to avoid excessive levels of condensation from forming on the lid, which would hinder visibility of the culture inside. The plates were then placed in an incubator at 25C° for four weeks.

7.4. Harvesting and Processing

After four weeks, an image of each plate was captured with an Epson Perfection V700 Photo scanner in 24-bit color at a resolution of 4800dpi. These images were used to conduct spore counts (**Images 3** and **4**). For this, two isolated square areas of each plate were chosen (in the center near the barrier, and in the center near the edge of the plate) and spores within these areas were counted manually. High magnification images of plates were also captured using a Leica M165C Stereoscope to better convey the details of the extraradical morphology.





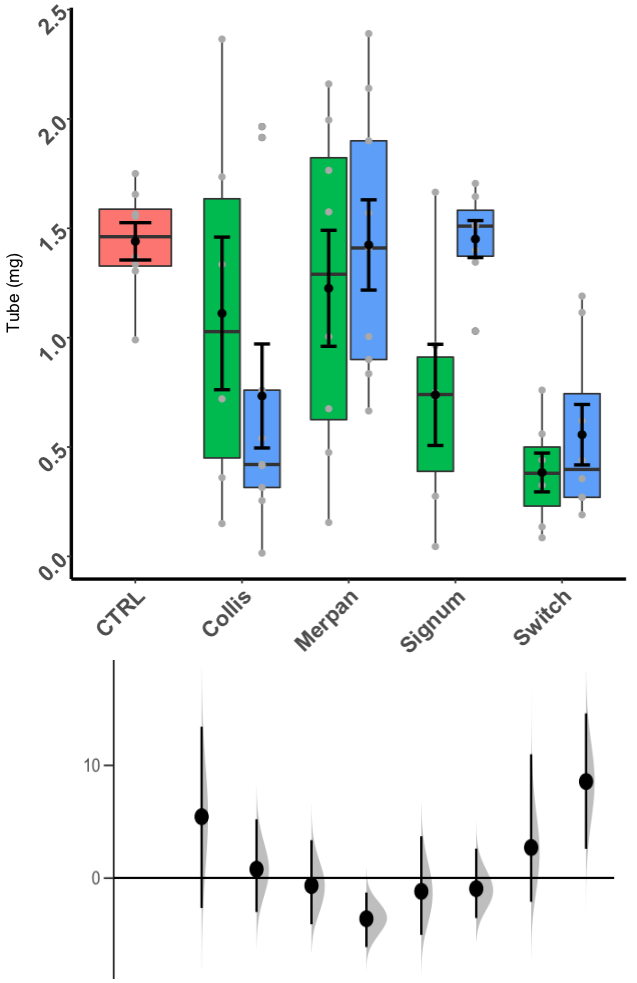
Images 3 (left) shows a higher magnification of the same area in **image 4** (right). The arrows indicate what were considered to be spores for the spore counting protocol. Only mature spores were counted - those round in shape, growing next to visible mycelium, relatively translucent and larger than other "specks" or immature spores present in the medium.

The mycelium and spores were then extracted from the plates. For this, a citrate solution was prepared using 12 grams of sodium citrate per liter of distilled water adjusted to a pH of 6 and heated to 60 C° to liquify the M-medium of the mycelium compartment. The liquified medium containing the spores and mycelium was then filtered through a vacuum pump to obtain the isolated wet biomass which was then placed into pre-weighed eppendorf tubes. These tubes were weighed (a second time) using a high precision balance. The tubes containing the wet biomass were then placed, open, in a drying oven at 40° for 3 hours. They were then weighed again to measure dry biomass.

8. Results

8.1. Extraradical Mycelial Biomass

The fungicide that most impacted mycelial biomass at both high and low doses was Switch, with little variation between both doses (**Figure 1**). Signum also had a significant impact on mycelial biomass but only at a high dose. No significant effect was observed for Captan. Collis significantly reduced biomass in the low dose. A 4-way Anova statistical test was carried out to evaluate the significance of each factor and their combinations (**Table 1**). Fungicide application was found to be statistically significant with a P value < 0.05.



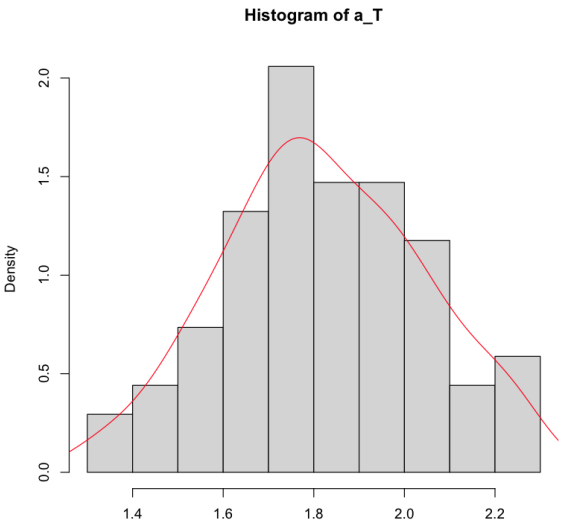


Figure 1 (left). Dry biomass (mg), including both mycelium and spores, of AMF exposed to high and low doses of four commercial fungicides. Below is a measurement of the effect size, revealing that only the low Merpan dose and high Switch dose differed significantly from control.

Figure 2 (right). Histogram of biomass measurements, which follow a normal distribution.

	Df	Sum Sq	Mean Sq	F value	Pr (> F)
Fungicide	4	0.5029	0.125725	2.8978	0.02942*
Dose	1	0.00012	0.000119	0.0027	0.95845
Fungicide: Dose	3	0.19098	0.063659	1.4673	0.23269
Residuals	59	2.55980	0.043386		

Table 1 - Anova statistical analysis; significance value : * = 0.05

8.2. Spore Production

The spore count revealed that the number of spores influenced the overall biomass as the distribution of each of the bars in the graph for the spore count roughly follows that of the biomass (**Figure 3)**. A noticeable effect can be seen for Merpan at both high and low doses which appear to have inhibited and stimulated spore production, respectively. For switch we can see a strong inhibition of spore production with respect to control. For Collis and Signum the effect appears not to have been particularly strong or regular, with the low signum dose leading to an increased spore production. A 4-way Anova statistical (**Table 2)** analysis was conducted which revealed a significant effect of fungicides on spore production as well as a dose dependent effect.

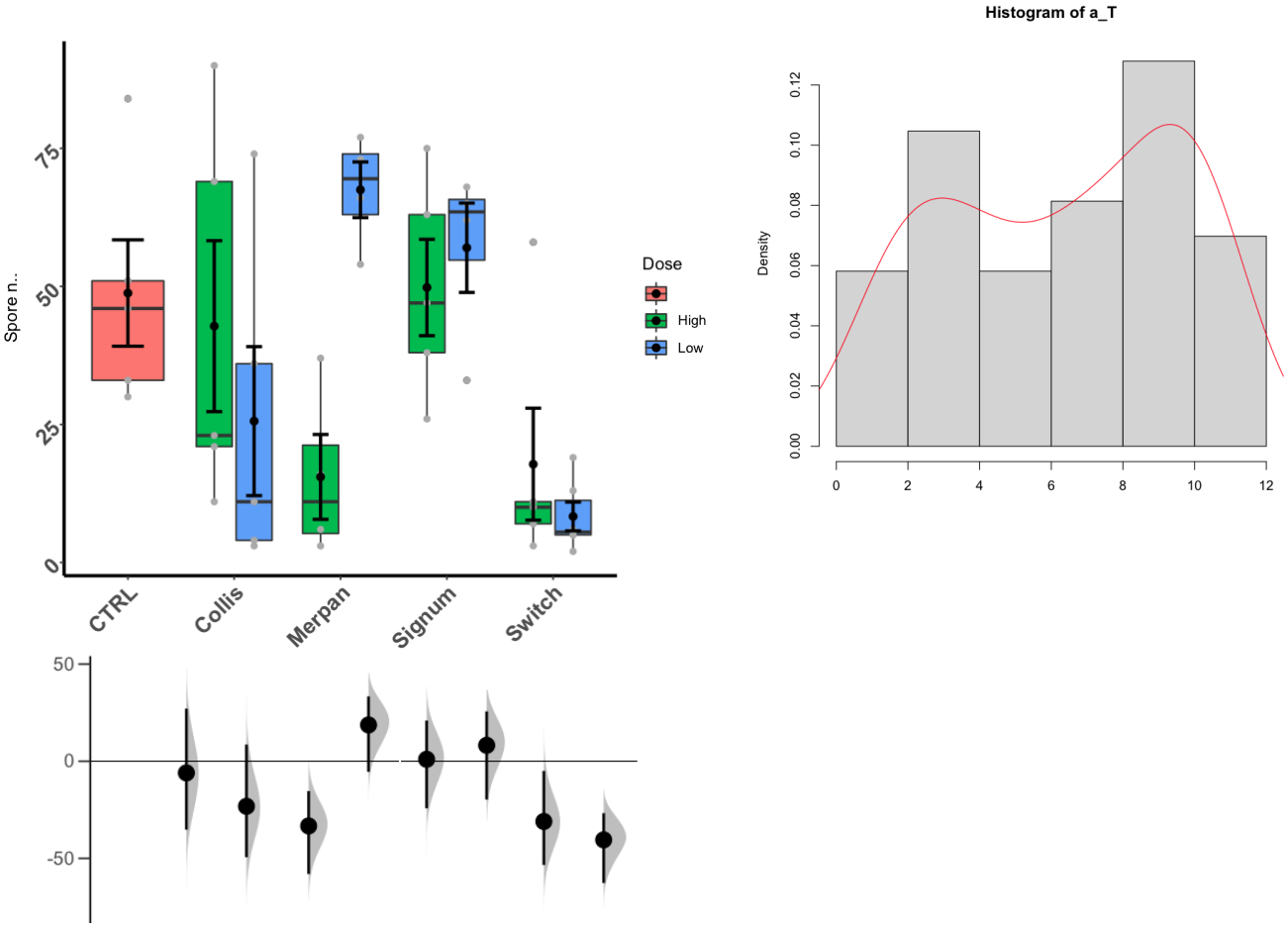


Figure 3 (left). Numbers of spores produced (per total counted area per plate) of AMF exposed to high and low doses of four commercial fungicides. Below is a measurement of the effect size, revealing that both doses of Merpan and the high Switch dose showed an effect that diverged significantly from control.

Figure 4 (right). Histogram showing the data shows a spread distribution.

	Df	Sum Sq	Mean Sq	F value	Pr (> F)
Fungicide	4	163.865	40.966	7.0401	0.0003055***
Dose	1	1.401	1.401	0.2408	0.6267468
Fungicide:Dose	3	86.287	28.762	4.9428	0.0059080 **
Residuals	34	197.845	5.819		

Table 2 - Anova statistical analysis; significance value : ** = 0.01; *** = 0.001

8.3. Extraradical Mycelium Architecture

8.3.1 Phenotype 1 : “Hairs”

Three abnormal phenotypes were noticed in the mycelium compartment. First, a number of hyphae were seen to be growing upwards, outside of the medium. This phenomenon occurred almost exclusively with the high treatments, in particular with Signum. No such “hairs” were noticed in the control group. The relative length of the hair displacing itself out of the medium can be noticed comparing image 1 and 2 below. Hyphae protruding from the medium measured 2-6mm. This phenomenon is highly uncharacteristic in AMF growing in M-medium and is to be distinguished from “aerial mycelium”, which typically occurs in networks as a normal part of vegetative or reproductive growth, while these “hairs” were all individual strands with no visible reproductive purpose.

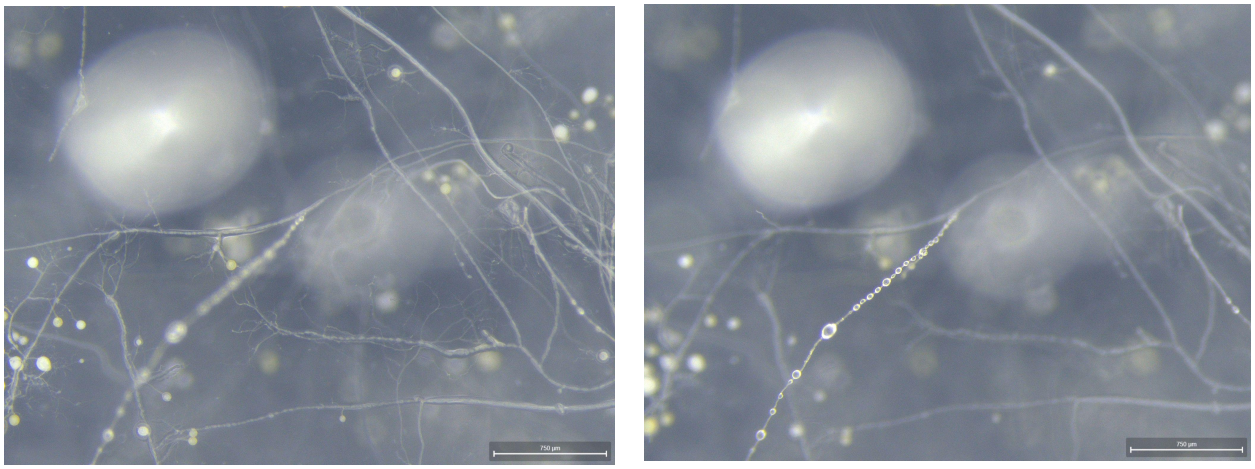
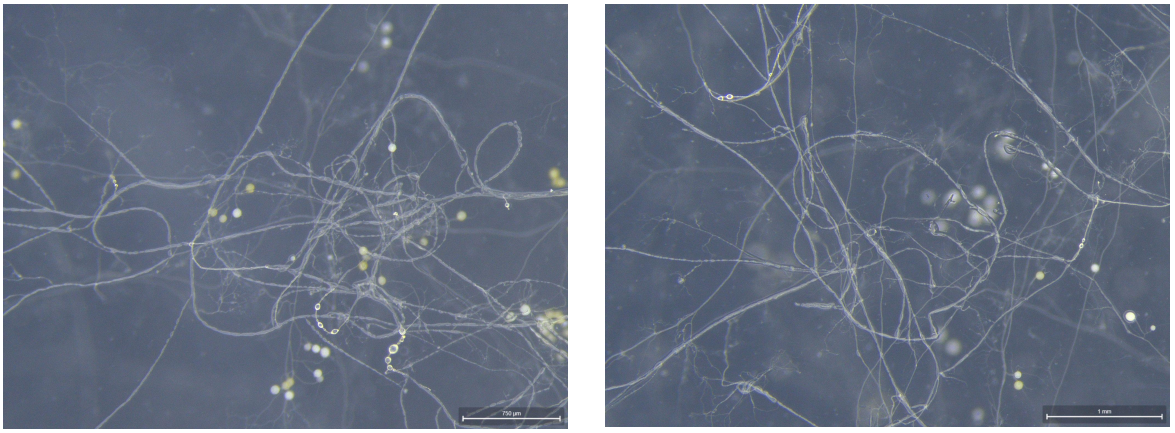


Image 1 (left) is in focus at the base of the “hair” and **image 2** (right) is in focus at its tip. This section of the hyphae was protruding nearly vertically out of the medium and droplets (unverified substance) were condensed around it.

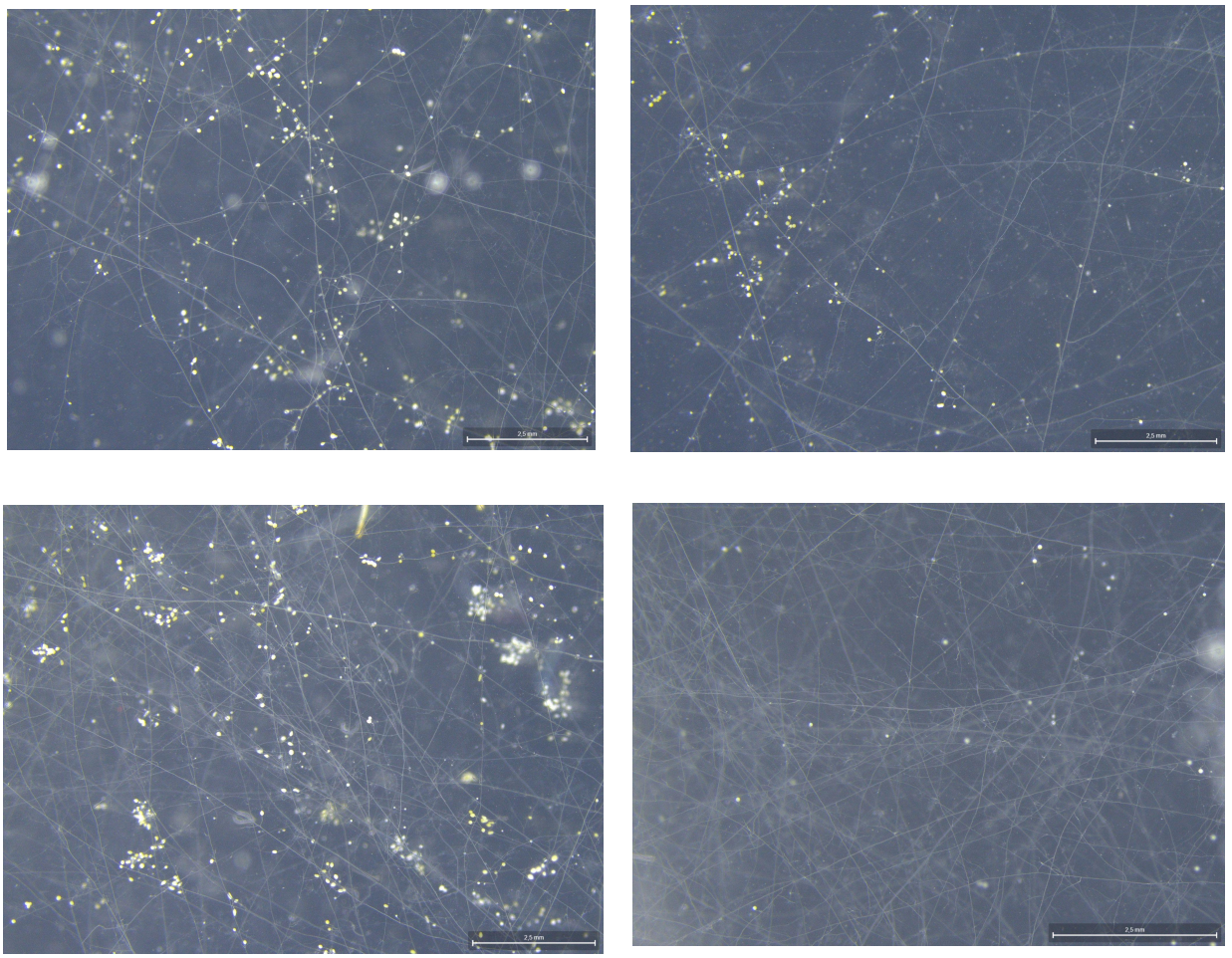
8.3.2 Phenotype 2 : Curvilinear Growth

The second abnormal phenotype that was noticed were contorted and anastomosis-like knots that formed among hyphae, which can be distinguished from the relatively straight hyphal architecture typically found with the control plates. **Images 5** and **6** show close up examples of this phenotype.



Images 5 (left) and **6** (right) are all from the high treatment with Signum**.** Such contorted phenotypes were seen in practically neither locations in low treatments nor in the control treatment, only in 2 plates from the high treatment with Collis and 4 plates with the high treatment with Switch.

These phenotypes can be contrasted with phenotypes typical of the control group. **Images 7**, **8, 9** and **10** display standard morphology, both for dense and sparse mycelial architectures, as well as for areas containing spores and not.

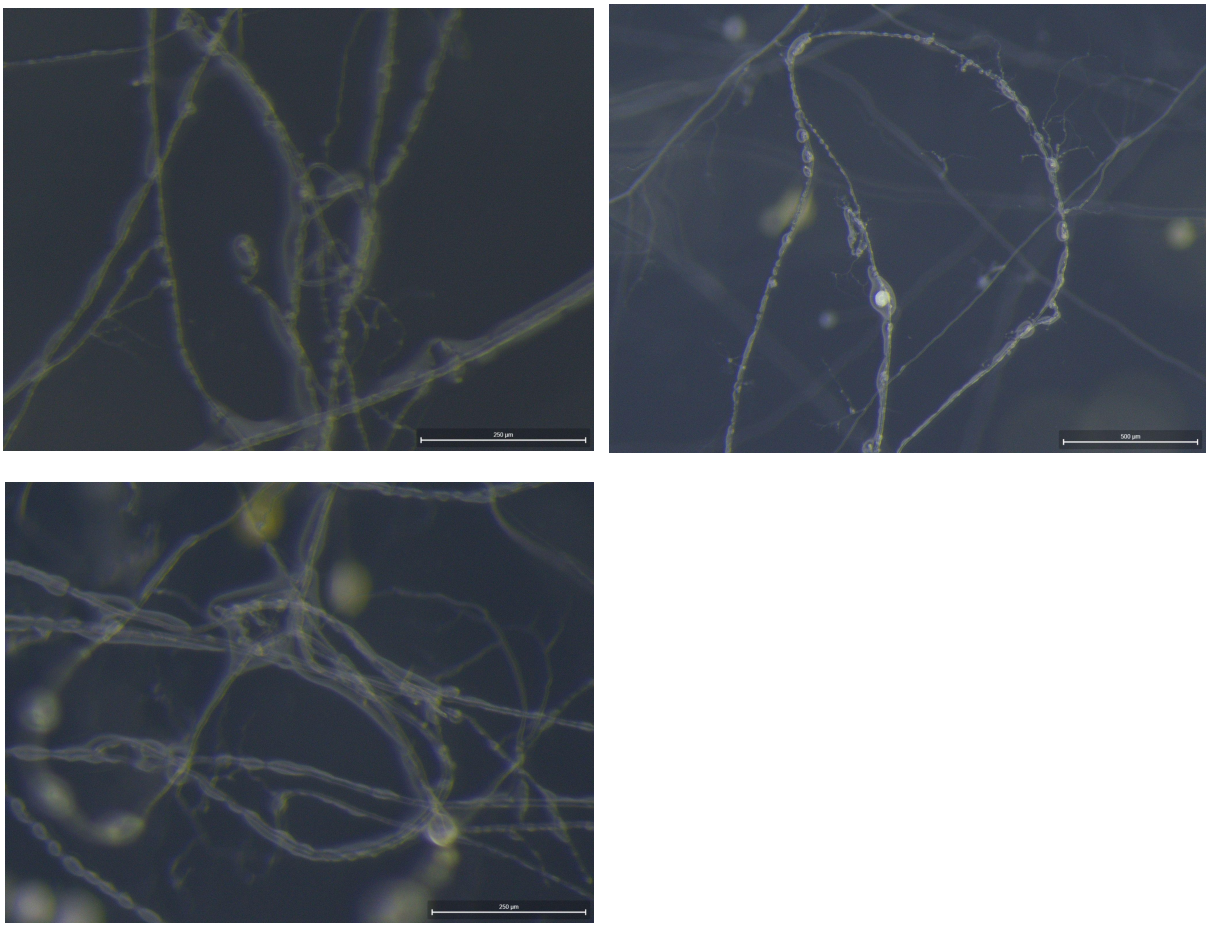


Images 7 (top left), **8** (top right), **9** (bottom left) and **10** (bottom right) show mycelium in which the direction of growth of the vast majority of hyphal segments occurs along trajectories that remain within an angle of at most 20° with respect to the trajectory of the segment prior the previous branching point. Few to no filaments ever fold back along themselves.

8.3.3 Phenotype 3 : Hyphal Exudates / Uneven Hyphal Width

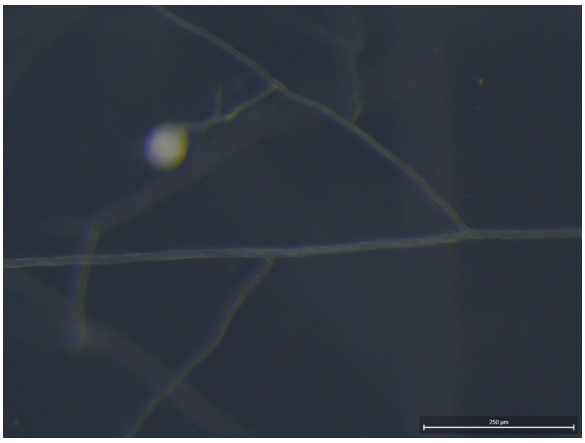
The third abnormal phenotype that was noticed was typically seen in conjunction with the curled phenotype mentioned above. In these cases, a type of corrosion, change in hyphal width, or exudation of a substance immediately around the hyphae was noticed. **Images 11, 12** and **13** are examples of this phenotype. Due to the translucency of hyphae, the exact nature of this phenotype was

unclear, whether it was the width of the hyphae itself that varied in size or if a substance was coating or surrounding the hyphae.



Images 11 (top left), **12** (top right) and **13** (bottom left) show a phenomenon occurring around the hyphae, within the gel substrate, that presented an unevenness in width. This was typically present in the contorted areas of phenotype 2.

This phenotype was never observed for hyphae within the control group, which, along with not "curling", were virtually always even in length. **Image 14** is an example of such a characteristic morphology of AMF hyphae growing in M-medium. In the control group and for the low dose treatments, hyphae tended only to vary in length at locations of high branching intensity, typically as precursors to spore formation, as can be seen here in **image 15**.



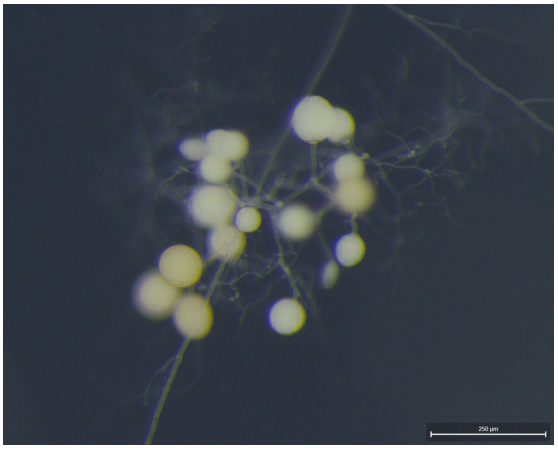


Image 14 (left) depicts a hyphae with a characteristically straight trajectory and without the appearance of any variation in width or the presence of a substance coalesced around it. **Image 15** (right) shows the only typical variation in hyphal length that occurred in areas of spore production.

9. Discussion

Since biomass was weighed including spores, spore number number had to be taken into account to extrapolate information from the mycelial biomass. In the case of Switch, for instance, a low biomass also correlated to a low spore count, and it is worth noting that instances of all three abnormal phenotypes were visible in AMF exposed to Switch, though much less than for Signum. Biomass measures were relatively close to control for the other fungicides, though tending towards a decrease with respect to control. When considering spores however, we can see that certain fungicides led to an increase in spore count with respect to control, which could signify a focus on spore production as opposed to mycelium production.

The capacity for mycorrhizal fungi to help alleviate salinity and heavy metal stress in plants is owed to their capacity to compartmentalize these substances in their vacuoles, but also to lock them away into spores. Not all their means of detoxifying complex compounds are known, but spore production could be a means by which they can sequester these compounds. Thus a higher spore count could have been the result of a detoxification procedure by the AMF.

Another factor that could have led to an increased spore count is a stress response leading the fungus to prioritize reproduction over growth. New spores have the potential to germinate after the toxic substance has naturally degraded in the spore's environment, while the stress caused by exposure to the fungicide could jeopardize the AMF's life and make reproduction more imperative. For Merpan the effect on spore count was particularly significant, with a relatively strong increase and decrease in spore count, respectively, for the low and high dose. We hypothesized that the low dose may have been enough to trigger a preemptive investment in Spore production, whereas the high dose may have been overpowering and inhibited spore production.

While the exact mode of action of these fungicides AMF is not fully clear, their capacity to combat pathogenic fungi by negatively affecting energy transduction (Collis), participating in the degradation of fungal cell walls (Merpan), growth rate (Switch) or dysregulating homeostasis (Signum) could explain their effect on fungal biomass and spore counts. Biomass, in and of itself, however, is not necessarily a strong indicator of performance. AMF exposed to Signum, for instance, showed no significant decrease in biomass but did display a particularly abnormal phenotype. The more homogenous increase in spore count between doses with respect to control that can be noticed with Signum may signal that the fungicide did not directly threaten the AMF's ability to grow and reproduce, but may have negatively affected it in other ways that could hinder its symbiotic potential.

We hypothesized that the three abnormal phenotypes noticed with the high Signum treatment had to do with detoxification or stress avoidance phenomena. The droplets visible around the hyphae could either be condensed water vapor, or exudates from the mycelium containing the fungicide as a result of cellular efflux of the fungicidal compound. The potential accumulation of triglycerides attributed to Signum may also have disrupted the structure of its cell walls. Given the relatively high humidity in the petri dish, we suppose another attempted strategy for dealing with the presence of the fungicide was avoidance by physical departure from the gel substrate. This could indicate that in some rare cases, the loss in nutritional benefits from the gel by moving outside of it was less costly than gaining distance from the fungicide in the medium.

Unlike spore production or the “hair” phenotype, the “curled” phenotype does not appear to be an adaptive mechanism but rather a growth defect induced by the fungicide. The curvilinear growth could be a result of a disruption of hyphal tip elongation in which the site of extension, the Spitzenkorper, is not able to function correctly, leading to a defective, curvilinear growth rather than a straight, elongating one. The only means we hypothesized by which this phenotype could confer an advantage would be through the seemingly higher rates of anastomosis visible in the “knot” structures, as such hyphal formations could allow the mycelium to more effectively displace the fungicidal compounds within itself.

9.1. Software-Based Image Analysis

A method we sought to use for this experiment but which did not succeed was a software-based image analysis using the Fungal Networks module, developed by Prof. Marck Fricker from the Department of Plant Sciences at Pembroke College, Oxord, within the Matlabs software. The purpose of this operation was to extract numerical data from images acquired by scanning our petri dishes, allowing for a quantification of parameters such as total hyphae length, number of hyphal branches, branching frequency, branching angle, number of anastomoses, number of hyphal tips, spore number, and so on and so forth. This was unfortunately not possible in this study due to time constraints along with the still early stage of the software and due to image quality constraints related to scanning features and condensation inside some plates. However, at this point in time, this software can effectively extract data in such a manner that can potentially lead to far quicker processes of measuring, counting and calculating than the current manual methods do for those parameters. With further development of the software as well as advances in CPU processing power, such a method has the potential to revolutionize not only AMF studies but studies of fungi altogether in response to any given biotic or abiotic factor. Below are the types of scans that could lend themselves to such image analyses.

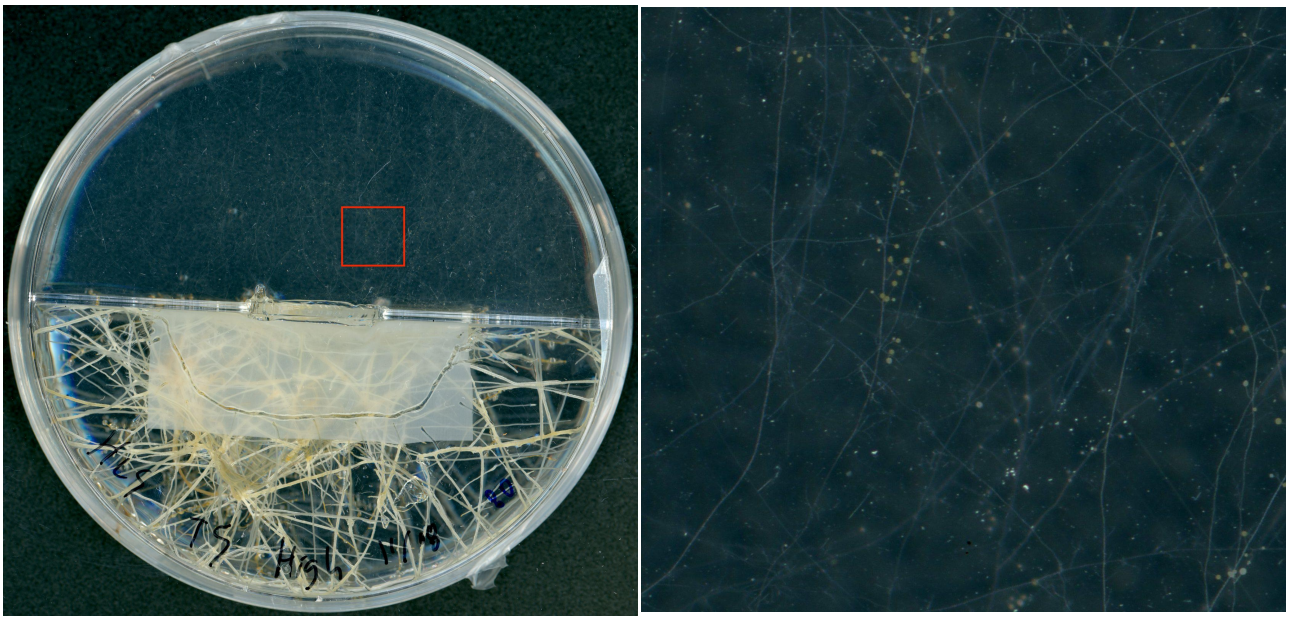


Image 16 (left) is a scan of a split-plate from the control group (resolution) and **image 17** (right) is a higher magnification of the zone in red from **image 16**. This plate exemplifies a typical growth pattern of the mycelium under normal conditions in M-medium. A scanned image of the sort could, under the right conditions, lend itself to software-based image analysis.

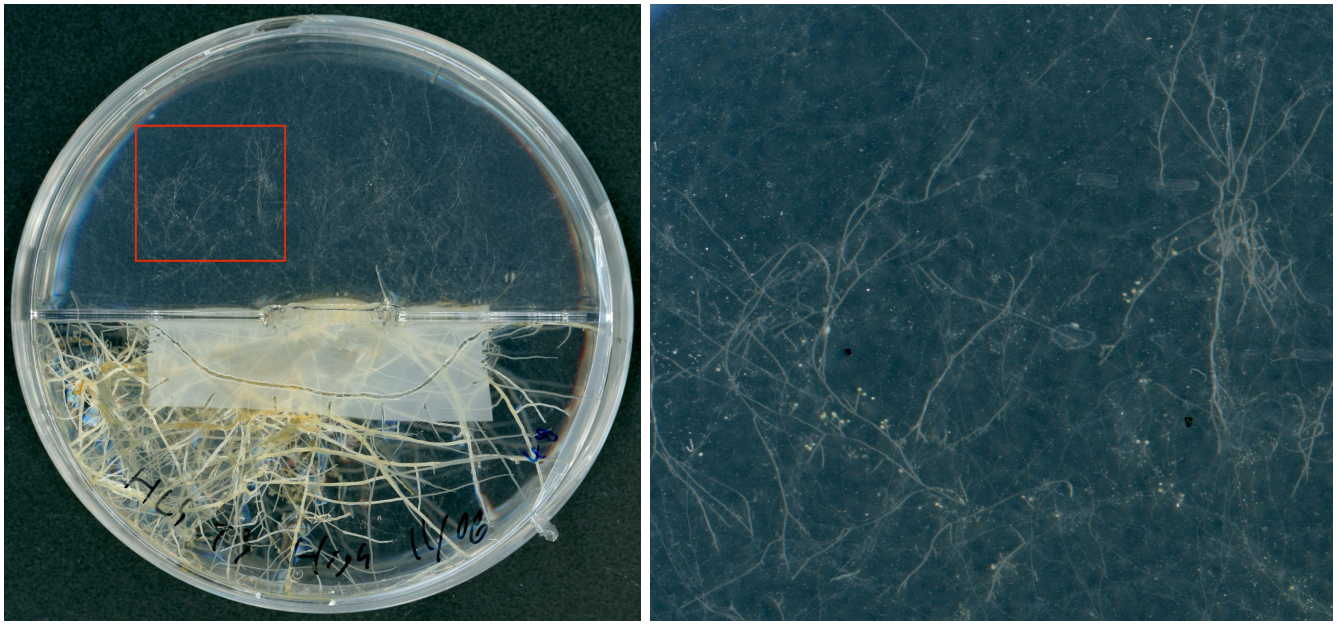


Image 18 (left) is a scan of a split-plate from the Signum high dose group. **Image 19** (right) is a higher magnification of the zone in red from **image 18**. This plate exemplifies an abnormal growth pattern of mycelium with curvilinear growth and irregular width. A scanned image of the sort could, under the right conditions, lend itself to software-based image analysis.

9.2. Petri Dish Modification

The potential which split plate root organ cultures allow is vast, and to conduct further studies on AMF on a more widespread scale, petri dish manufacturers could incorporate a more elaborate dual compartment system in their split plates so that modifications of the plate, such as those carried out in this experiment, do not fall into the hands of researchers who would greatly benefit from using pre-modified dishes instead of having to do the modifications themselves.

10. Conclusions

The results from this study do not indicate any type of compatibility between *R. irregularis* and the four commercial fungicides applied, except for a potential slight tolerance with regards to Collis. For the other three compounds, the combined impact on AMF biomass, spore counts and morphology tend to indicate a stress condition. Further metabolic and proteomic studies, however, are warranted to better understand how these fungicides affect AMF, how AMF detoxify them and how their effect on AMF would translate to an effect on host plants. As far as the initial steps made in this study are concerned, further investigation into the nature of the abnormal phenotypes, such as what led to the contorted growth or what the content of the substance was coating the mycelium in those contorted areas or the contents of the droplets on the hairs of the mycelium that had exited the gel would be of great interest.

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