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Impact of pesticides on arbuscular mycorrhizal fungi with contrasting life history strategies

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Fungi are not animals or plants; these organisms belong in their own kingdom. I am sure they must be the rarest organisms in the world (Miguel Rodriguez. Personal communication, Alamo, Mexico 199?).

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List of Abbreviations

a.i.	Active Ingredient
AM	Arbuscular Mycorrhizae
ANOVA	Analysis of Variance
AZO	Azoxystrobin
CA	Control Acetone
CC	Culture Compartment
CONACyT	Consejo Nacional de Ciencia y Tecnología [National Council of Science and Technology]
DAOM	Department of Agriculture, Ottawa, Mycology
ELIM	Earth and Life Institute - Microbiology
ERM	Extraradical Mycelium
GHTs	Growing Hyphal Tips
GINCO	Glomeromycota *in vitro* Collection
HL	Hyphal Length
hr	Hazard Ratio's
HHM	Hyphal Healing Mechanism
LHS	Life History Strategies
MC	Mycelial Compartment
MUCL	Mycothèque de l'Université Catholique de Louvain
MSR	Modified Strullu-Romand
Pi	Inorganic phosphorus
ROC	Root Organ Culture
RDW	Root Dry Weight
SDW	Shoot Dry Weight
TDW	Tuber Dry Weight

Glossary

Anastomosis: is the process of fusion between branches of the same or different hyphae to produce a mycelial network. It is a very common and important mechanism in the fungal kingdom (Glass et al. 2000; Kirk et al. 2001).

Arbuscule: highly branched haustoria-like structures formed in cortical root cells. Arbuscules are the centers of the bio-directional nutrient exchange between the AMF and the host. (Kokkoris et al. 2020).

Arbuscular mycorrhiza (AM) fungi: a symbiosis between plants and members of an ancient phylum of fungi, the Glomeromycota. They improve the supply of water and nutrients, such as phosphate and nitrogen, to the host plant. In return, plant-fixed carbon is transferred to the fungi. Nutrient transport occurs through symbiotic structures inside plant root cells known as arbuscules (Parniske 2008).

Branched absorbing structures: small groups of dichotomous hyphae formed by the ERM of AM fungi that play an important role in nutrient absorption (Bago et al. 1998).

Extraradical Mycelium (ERM): is a network of fungal hyphae that extends from the roots of mycorrhizal plants into the soil. ERM is a key component of the symbiosis with many important roles, including fungal development, nutrient/water transport, allows root colonization mycelial network and form mycelial network (Malcová et al. 2001).

Fungicide: is any substance, preparation, or organism intended for destroying or controlling any fungal species during production, storage, or distribution of an agricultural commodity or food, in ornamental plants, or in situations endangering the health of animals or humans (Smart 2003).

Herbicides: is any individual substance or mixture that is intended to prevent, control, destroy, repel, or mitigate growth of unwanted plants or parts of them (Vasilescu and Medvedovici 2005).

Hormesis: an adaptive response widely observed in various organisms. This phenomenon occurs when exposure to low doses of a stressor potentially induces a stimulatory effect, while higher doses may inhibit it (Wan et al. 2014).

Hyphal healing mechanism: formation of hyphal bridges to reconnect broken hyphae or overcoming obstructed areas within a hypha (de Souza and Declerck 2003).

Hyphopodium: is a lobed hyphal contact point with the root that serves as the entry point of the fungus into the epidermis. The hyphopodium is a specialized structure functionally similar to the fungal pathogenic appressorium (Murray et

al. 2013).

K-strategists: species that invest more in offspring quality than quantity, resulting in slow population growth. Stable ecosystems favor the evolution of K-selected species (Cassill 2019).

Life history strategy: patterns of resource allocation in which organisms optimise their lifetime fitness according to the trade-off between reproduction, growth, and survival (Wang et al. 2018).

Pesticides: are chemical compounds that are used to kill pests, including insects, rodents, fungi, and unwanted plants. Over 1000 different pesticides are used around the world. Pesticides are used in public health to kill vectors of disease, such as mosquitoes, and in agriculture to kill pests that damage crops (World Health Organization 2020).

r-strategist: species that invest more in offspring quantity than quality, resulting in rapid population growth. Unstable ecosystems favor the evolution of r-selected species (Cassill 2019).

Runner hyphae: type of extraradical hyphae produced by AM fungi after colonization that grow into the soil and subsequently produce branched absorbing structures to take up P and micronutrients, which are transferred to the plant (Bago et al. 1998).

Spitzenkörper: is a dynamic structure present at the tips of hyphal cells with a single highly polarized growth site. It is closely connected with cell morphogenesis and polar growth and is only present at actively growing sites (Virag and Harris 2006).

Vesicles: lipid-rich fungal storage organs that are formed intercellularly within the cortex when the carbon supply is sufficient (Schaarschmidt et al. 2007).

Summary

Pesticides are widely used in crop production, mostly to control weeds (herbicides) and fungal diseases (fungicides). However, non-target beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi, may also be affected by these compounds, both directly by contact with the active substances or indirectly by changes in the host plant physiology. In this context, the general objective of the thesis was to study the impact of four fungicides (azoxystrobin, pencycuron, flutolanil and fenpropimorph) and the world-largest used herbicide (glyphosate) on hyphal healing mechanism (HHM) of *Rhizophagus irregularis* MUCL 41833 (*Glomeraceae*) and *Gigaspora* sp. MUCL 52331 (*Gigasporaceae*). The impact of fungicides was evaluated on anastomosis process in intact mycelial networks *R. irregularis* as well as the dynamics of phosphorus uptake. These AM fungi belong to phylogenetically distant families and have contrasting life history strategies (LHS). The *Glomeraceae* appears more adapted to agro-ecosystems than the Gigasporaceae which are more abundant in undisturbed ecosystems. Different studies were conducted both *in vitro* and under semi-hydroponic cultivation conditions.

Results suggested that fungicides with different modes of action and concentrations (0.02 and 2 mg L^{-1}) as well as glyphosate at increasing concentrations (0.02, 0.2 and 2 mg L^{-1}) impact differentially the anastomosis and HHM processes. The systemic fungicide azoxystrobin at 2 mg L^{-1} was particularly detrimental to the HHM in both AM fungi. Curiously, fenpropimorph, another systemic fungicide, only impacted *R. irregularis*, stimulating at low and inhibiting at high concentration the HHM. Additionally, glyphosate at 2 mg L^{-1}, stimulated the emission and contact of growing hyphal tips (GHT) during HHM in *R. irregularis*, while no impact was noticed in *Gigaspora* sp. The effects of fungicides on the anastomosis formation within and between networks in intact mycelial networks of *R. irregularis* also varied according to the fungicide. Azoxystrobin and fenpropimorph had a particularly detrimental impact, at the highest concentration (2 mg L^{-1}), on the number of anastomoses within and between networks. In contrast, flutolanil and pencycuron did not impact the anastomosis process in the two AM fungi. These results suggest that pesticides with different modes of action and concentrations differentially affect anastomosis and HHM in AM fungi with contrasting LHS and may thus affect the capacity of AM fungi to maintain and develop mycelial networks.

Finally, a semi-hydroponic cultivation system was used with potato plants colonized or not by *R. irregularis* MUCL 41833 to investigate the dynamics of inorganic phosphorus (Pi) uptake in presence/absence of azoxystrobin. This fungicide decreased Pi uptake by plants after 30 days of growth in presence as well as in absence of the AM fungus. Conversely, after 60 days in absence of azoxystrobin, a larger Pi uptake was measured in the mycorrhizal plants, while the reverse was noticed in presence of azoxystrobin. The mycorrhizal plants were

more affected by azoxystrobin than those growing in absence of the fungicide, suggesting a direct effect of azoxystrobin on *R. irregularis* and an indirect effect on the symbionts via a possible imbalance in the transfer of nutrients.

Keywords

Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* MUCL 41833, *Gigaspora* sp. MUCL 52331, Anastomosis, Hyphal Healing Mechanism, Fungicides, Herbicides, *Solanum tuberosum*.

Outline of the thesis

Within this thesis, different experiments were conducted to address four main questions:

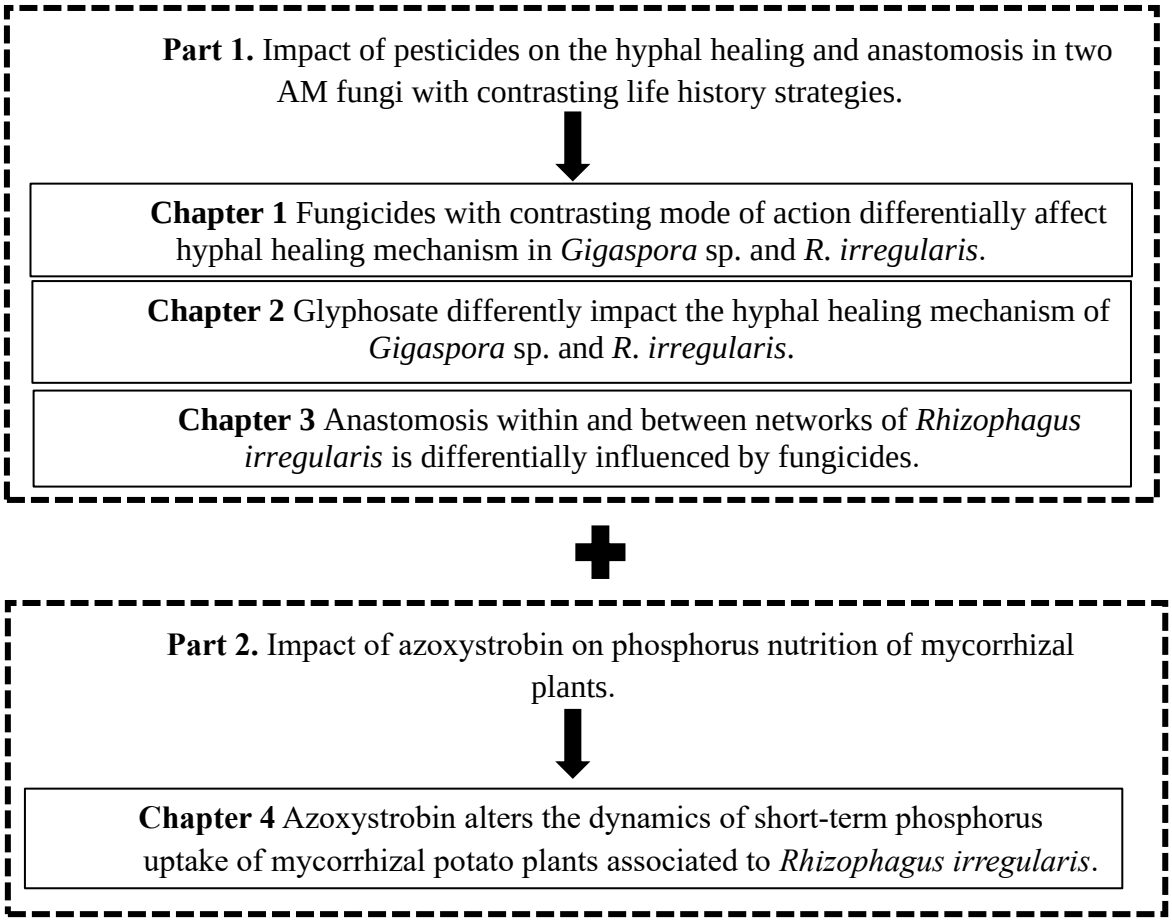
1. What is the impact of different classes of fungicides on the hyphal healing mechanism (HHM) in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL52331, two AM fungi with different life history strategies?

2. What is the impact of the herbicide glyphosate on the HHM in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331?

3. How does fungicides with contrasting mode of action affect the anastomosis process within and between different networks of *R. irregularis* MUCL 41833?

4. Is the dynamics of Pi uptake by potato plants grown in presence of the fungicide azoxystrobin more efficient when associated to *R. irregularis* MUCL 41833 than in absence of the fungus?

The successive steps to answer these questions are schematized as follow:



The thesis is divided in two majors parts with the **general objective** to investigate the impact of fungicides and herbicides on (**Part 1**) the hyphal healing mechanism (HHM) and anastomosis process in AM fungal species belonging to the *Glomeraceae* and *Gigasporaceae*, two major phyla of the Glomeromycota having contrasting life history strategies (LHS) and (**Part 2**) the dynamics of Pi uptake by *Rhizophagus irregularis* MUCL 41833 in presence of azoxystrobin, a major fungicide used in potato crops to control *Rhizoctonia solani*.

In the **Introduction** section, the context and the general objectives of the thesis are presented. In the **State of the Art** section, an exhaustive bibliography review is done on the impact of the most important pesticides used in crops (e.g., fungicides and herbicides) on AM fungi. The review also includes a description of the extraradical mycelium and hyphal healing and anastomosis processes in AM fungi.

The **Materials and Methods** section details the biological material and provides a wide description of the most important techniques used throughout the thesis.

The research **Results** section is divided in two parts. The first part contains three chapters and is strictly conducted with root organ cultures. It is aimed at investigating the impact of different classes of widely-used pesticides (e.g., fungicides, herbicides) on the HHM, anastomosis process and spore germination using two AM fungal strains *Gigaspora* sp. MUCL 52331 (*Gigasporaceae*) and *Rhizophagus irregularis* MUCL 41833 (*Glomeraceae*), with contrasting LHS. The second part contains a single chapter and is conducted with potato plants grown in the greenhouse. It is aimed at investigating the dynamics of Pi uptake by mycorrhizal plants in presence of the fungicide azoxystrobinhe objective of **Chapter 1** was to investigate under *in vitro* culture conditions the impact of azoxystrobin, pencycuron, flutolanil, and fenpropimorph at two different concentrations (0.02 and 2 mg L^{-1}) on the HHM of *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833, belonging to phylogenetically distant Glomeromycota families and thus having different LHS.

We described and compared the events conducting to a full recovery using a dissecting bright-field light microscope at defined time intervals.

The results of Chapter 1 were published in 'Frontiers in Plant Sciences' (Rodriguez-Morelos et al. 2021).

The objective of **Chapter 2** was to investigate under *in vitro* culture conditions the impact of glyphosate at increasing concentrations (i.e., 0.02, 0.2 and 2 mg L^{-1} a.i.) on the HHM of *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833.

Similarly, to **Chapter 1**, we described and compared the events conducting to a full recovery using a dissecting bright-field light microscope at defined time intervals.

The results of Chapter 2 are in preparation for submission to 'Mycorrhiza'.

The objective of **Chapter 3** was to investigate under *in vitro* culture conditions the impact of azoxystrobin, pencycuron, flutolanil, and fenpropimorph at two concentrations (0.02 and 2 mg L^{-1} a.i.) on the capacity of *R. irregularis* to form anastomoses within and between hyphal networks of the same isolate of *R. irregularis* MUCL 41833.

The results of Chapter 3 were published in 'Mycorrhiza' (Rodriguez-Morelos et al. 2023).

Finally, the objective of **Chapter 4** was to evaluate under greenhouse conditions the dynamics of Pi uptake of potato plants associated to *R. irregularis* MUCL 41833 and grown in presence or absence of azoxystrobin. The study was conducted non-destructively under a circulatory semi-hydroponic cultivation system connecting each plant to a nutrient solution. The depletion of Pi was measured at regular intervals in the nutrient solution flowing on the plant roots, thus indirectly evaluating the Pi uptake by the plant/AM fungus associates.

The results of Chapter 4 were published in 'Journal of Plant Nutrition and Soil Science' (Rodriguez-Morelos et al. 2022).

In the **General Discussion** section, the overall results of the thesis are summarized and discussed. In the **Conclusions and Perspectives** section, the most important findings and prospects for the study of pesticides interactions with AM fungi are presented.

Author's contribution

The work presented here was realized during the time course of my PhD.

The introduction, state of the art, general discussion, conclusions and perspectives were written by myself and were not considered for publication.

Chapter I is a research paper published in **Frontiers in Plant Sciences** (2021). My contribution to this paper was estimated to 75%. The *in vitro* cultures, data collection and writing of the manuscript were conducted by myself. The statistical data analysis with R software was performed with the help of Vincent Bremhorst (Statistical Methodology and Computing Service, technological platform at UCLouvain – SMCS/LIDAM, UCLouvain).

Chapter II is a short note in preparation for submission to **Mycorrhiza**. My contribution to this chapter was approximately 75%. The *in vitro* cultures, data collection, statistical analysis and writing of the paper were conducted by myself.

Chapter III is a short note published in **Mycorrhiza** (2023). My contribution to this chapter was approximately 75%. The *in vitro* cultures, data collection and writing of the paper were conducted by myself. The data analysis with R software was performed with the help of Vincent Bremhorst (Statistical Methodology and Computing Service, technological platform at UCLouvain – SMCS/LIDAM, UCLouvain).

Chapter IV is a research paper published in **Journal of Plant Nutrition and Soil Science** (2022). My contribution to this paper was estimated to 75%. The experiment setup, data collection, and writing of the manuscript were conducted by myself. François Ferrais (Technician at Louvain, Earth and Life Institute - Applied Microbiology - Laboratory of Mycology) helped for experiment setup and harvest. The ICP-AES analysis of the samples was performed by Anne Iserentant (Technician at Environmental Sciences, Earth and Life Institute, UCLouvain). The statistical analysis with R software was performed with the help of Alain Guillet and Benjamin Colling (Statistical Methodology and Computing Service, technological platform at UCLouvain – SMCS/LIDAM, UCLouvain)

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I. INTRODUCTION

Pesticides or plant protection products are used worldwide to control various pests and diseases in crops such as weeds (herbicides), fungi (fungicides) and insects (insecticides) (Cullen et al. 2019). Herbicides and fungicides are the most used pesticides and together with the improvement in crop varieties, have considerably increased crop yields and improved product quality (Gupta 2017). Although these plant protection products have many benefits for agriculture and food production (Cooper and Dobson 2007), there are a number of potential risks associated with their use (Cullen et al. 2019). These potential risks include pest resistance, environmental pollution, and human health hazards as well as impact on non-target micro- and macro-organisms and ecosystems unbalance (Kim et al. 2017; Lammoglia et al. 2017; Kalyabina et al. 2021).

Pesticide application can impact directly (e.g., by contact with the active substances) or indirectly (e.g., by changing the host plant physiology) non-target beneficial soil microorganisms (e.g., fungi, bacteria, algae, protozoa), which for some, are crucial for plant health and soil fertility in agroecosystems. Among these microorganisms, arbuscular mycorrhizal (AM) fungi (phylum Glomeromycota) are an important group of soil fungi forming symbiotic associations with over two thirds of vascular plants, including most crop species (Brundrett and Tedersoo 2018). The basis of this symbiosis lies in the supply of phosphorus (P) (Ferrol et al. 2019) and nitrogen (N) (Xie et al. 2022) by the fungi to plants in exchange for carbon sources, fixed during photosynthesis, by the plants to fungi (Keymer et al. 2017). A number of studies have also demonstrated that the AM symbiosis can increase the tolerance of plants to environmental stresses (e.g., drought, salinity, pollutants and pathogens) (Lenoir et al. 2016; Plouznikoff et al. 2016; Begum et al. 2019; Diagne et al. 2020) and even play major roles in soil structure via the production of glomalin (Wu et al. 2015). Nowadays, growing evidence indicates that these symbiotic microorganisms are a potential option to reduce the incidence of plant pathogens through an integrated control strategy allowing to limit the use of pesticides in crops (Ismail et al. 2013; Schouteden et al. 2015; Eke et al. 2016; Fiorilli et al. 2018; Boutaj et al. 2022).

Interestingly, AM fungi form large mycelial webs in the soil by connecting the roots of plants belonging to the same or different species (Bücking et al. 2016) via a complex and extensive network of extraradical mycelium (ERM), which overgrows the nutrient depletion zone of the soil surrounding plant roots, thus enhancing nutrients uptake (Pepe et al. 2018; Ferrol et al. 2019). This ERM is able to interconnect different branches of the same or different hyphae to constitute a mycelial network through anastomosis (Giovannetti et al. 2004; Voets et al. 2006; de Novais et al. 2019a, 2019b). The anastomosis in fungi is defined as: "*The fusion between branches of the same or different hyphae to produce a mycelial network, that involve degradation of the cell wall, followed by plasma membrane fusion*" (Glass and Fleissner 2006). In particular, the anastomosis in AM fungi, allows the mycelial growth and the physical exploration of the environment forming a three-dimensional network with protoplasmic/cytoplasmic flow, genetic material exchange and nutrients flow (Giovannetti et al. 2001; Mikkelsen et al. 2008; Croll et al. 2009; Giovannetti et al. 2015; Daubois et al. 2016; de Novais et al. 2017; Sbrana et al. 2018). Under *in vitro* culture conditions, de la Providencia et al. (2005, 2007) and de Novais et al. (2013) demonstrated that AM fungal species belonging to *Glomeraceae* and *Gigasporaceae* differed in pattern and densities of anastomosis. For example, hyphal networks of colonies belonging to the same isolate were reported to interconnect in the *Glomeraceae*, while in the *Gigasporaceae* such process was never observed (Voets et al. 2006). The anastomosis process also concerns recovery of mycelial network

integrity after physical damage. This specific process was described *in vitro* as a hyphal healing mechanism (HHM) (de la Providencia et al. 2007). Importantly, the HHM also differs between *Glomeraceae* and *Gigasporaceae*. Indeed, the HHM in *Glomeraceae* is oriented towards the reconnection of the affected area by linking several hyphae in relatively small vicinity or by recolonization of roots and substrate, by contrast to *Gigasporaceae* in which the HHM is nearly always oriented towards the re-establishment of hyphal integrity (de la Providencia et al. 2007). The different responses to physical disturbances suggest that both groups have developed different strategies to survive and colonize plants under adverse conditions (de la Providencia et al. 2007). Curiously, *Gigasporacea* tend to disappear in agricultural soils, while *Glomeraceae* seems more prevalent under cultural practices such as chemical fertilization, plowing and long-term application of pesticides (Jansa et al. 2003; Antunes et al. 2006; Soka and Ritchie 2018). In general, several studies have reported variable effects of fungicides and herbicides acrive ingredients and formulated products on AM fungi. However, knowledge on the impact of these pesticides on the process of anastomosis of ERM is scarce and is totally unknown for HHM, while it could drastically reduce the fungal networking and efficacy in nutrients transport, especially P, plant-to-plant interconnections, etc.

The general objective of the thesis is to investigate the impact of fungicides and herbicides on the growth and physiology of *Glomeraceae* and *Gigasporaceae*, two major groups of arbuscular mycorrhizal fungi having contrasting life history strategies (LHS). To achieve this objective, different studies were performed *in vitro* and under semi-hydroponic cultivation conditions. A first study was aimed to investigate the impact of azoxystrobin, pencycuron, flutolanil, and fenpropimorph on the HHM of two AM fungi (*Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833) belonging to phylogenetically distant families with different LHS. A second study was conducted to investigate the impact of glyphosate (the world's most widely used herbicide) on the HHM in *Gigaspora* sp. and *R. irregularis*. Subsequently, a third study was conducted to assess the impact of azoxystrobin, pencycuron, flutolanil, and fenpropimorph on the capacity of *R. irregularis* to form anastomoses within and between hyphal networks of the same isolate. We hypothesized that *Glomeraceae* and *Gigasporaceae* differ in their resistance/tolerance to fungicides and herbicides and that these differences are possibly related to LHS. More precisely, we postulate that anastomoses formation, HHM and development of ERM drastically differ between both families under pesticide exposure, explaining partly why the *Glomeraceae* are more prevalent in agricultural systems as compared to the *Gigasporaceae*, that tend to disappear.

Noticeably, the disruption of mycelial networks in the presence of pesticides could alter the dynamics of phosphorus uptake and reduce the benefits of the symbiosis for plant growth. Therefore, as a corollary, a fourth study was designed to evaluate the dynamics of Pi uptake of potato plants associated or not to *R. irregularis* and growing in presence or absence of azoxystrobin. This study was conducted non-destructively in a circulatory semi-hydroponic cultivation system connecting each plant to a Hoagland low-P solution under greenhouse conditions.

II. STATE OF THE ART

II.1. Pesticides

The Food and Agriculture Organization of the United Nations defined a pesticide as "*any substance intended for preventing, destroying, attracting, repelling or controlling any pest including unwanted species of plants or animals during the production, storage, transport, distribution and processing of food, agricultural commodities, or animal feeds or which may be administered to animals for the control of ectoparasites. The term includes substance intended for use as a plant growth regulator, defoliant, desiccant, fruit thinning agent, or sprouting inhibitor and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport*". Pesticide is thus a complex term that encompasses all compounds that are applied to destroy or regulate pests (Hassaan and El Nemr 2020). Pesticides, also named plant protection products, can also be defined as compounds that "*protect crops or desirable or useful plants. They are primarily used in the agricultural sector but also in forestry, horticulture, amenity areas and in home gardens. They contain at least one active substance and have one of the following functions: 1) protect plants or plant products against pests/diseases, before or after harvest; 2) influence the life processes of plants (such as substances influencing their growth, excluding nutrients); 3) preserve plant products; 4) destroy or prevent growth of undesired plants or parts of plants"* (European Commission 2022).

Pesticides can be classified according to their chemical nature into organic and inorganic compounds:

- **Organic pesticides** are based on chemicals that have carbon as the basis of their molecular structure. Oxygen, phosphorus or sulfur can also be inserted into their molecules. These pesticides can be further divided into natural or synthetic compounds. Natural organic pesticides are derived from naturally occurring sources such as plants, while synthetic organic pesticides or 'modern pesticides' are produced artificially by chemical synthesis (Urkude et al. 2019).

- **Inorganic pesticides** are substances derived or refined from non-living natural sources. They do not contain carbon compounds. Copper and sulphur-based fungicides are the main inorganic pesticides used to control pests and diseases (Karuppuchamy and Venugopal 2016).

Pesticides can also be classified according to the target organisms (Li and Jennings 2017), with the most important being herbicides, fungicides, and insecticides, to control weeds, fungi and insects, respectively (De et al. 2014; Tadeo 2019).

Finally, pesticides can be classified based on their chemical type (e.g., carbamates, organophosphates, organochlorines, pyrethroids), their mode of translocation in plants (i.e., systemic, local systemic or translaminar, contact), mode of action (e.g., impact on the cytoskeleton and motor protein, respiration) (Sharma et al. 2019; Hassaan and El Nemr 2020), and by hazard to human health (from extremely hazardous to unlike to present acute hazard (World Health Organization 2020).

Nowadays, conventional pesticides (i.e., synthetic chemicals or agrochemicals) are the most widely used compounds for the management of agricultural pests and diseases to increase

quality and quantity of yields, prevent vector-borne diseases and to generate profit (Yadav et al. 2015; Abubakar et al. 2020). The usage of pesticides increased by more than 50 percent in 2010 compared to 1990 (Rasool et al. 2022). However, its use has remained constant in the period 2010 to 2019. For example, in 2019, the global usage of pesticides has been estimated to 4.2 million metric tons (Food and Agriculture Organization Corporate Statistical Database 2021), mainly divided into herbicides (53%), fungicides (23.1%), insecticides (16.7%) and 7% of other biocides (**Figure 1**).

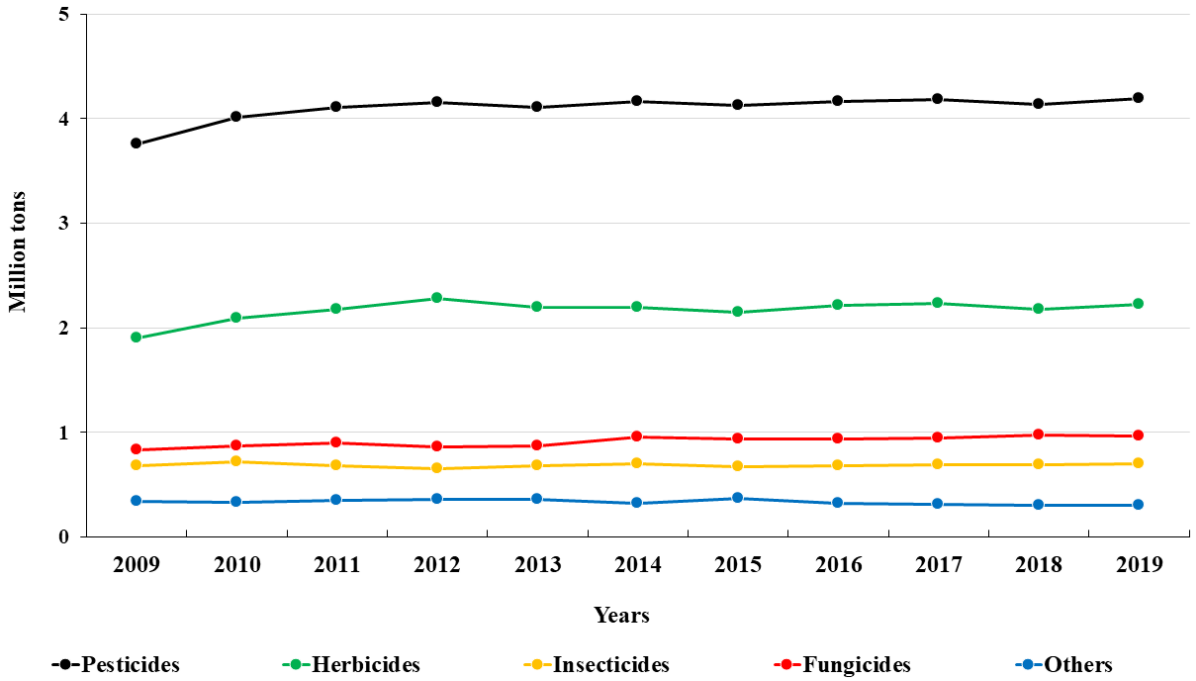


Fig. 1. Global usage of pesticides over the period 2009-2019 (Food and Agriculture Organization Corporate Statistical Database 2021).

During last decade, China, USA and Brazil were the largest pesticide users in the world. Remarkably, China was by far the largest pesticide-consuming country (1.46 million metric tons), followed by the United States (408 thousand tons), Brazil (377 thousand tons), and Argentina (204 thousand tons) (**Figure 2**).

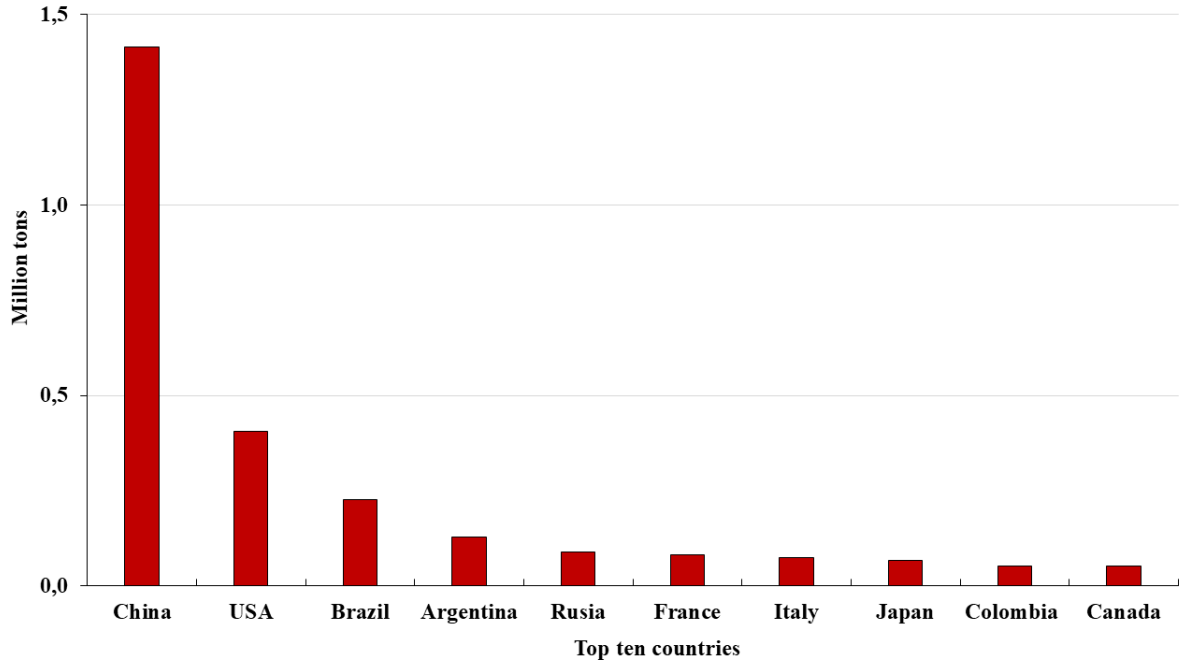


Fig. 2. Average pesticide use (1990-2019) in the top ten of consumers countries (Food and Agriculture Organization Corporate Statistical Database 2021).

II.1.1. Pesticides of interest

In this thesis, five different pesticides (four fungicides and one herbicide) were selected due to their various mode of action, their intensive use at the start of the thesis, and their used on the main crops (potato, wheat, beet, rice) (**Table 1**).

Table 1. Pesticides considered in the thesis classified according to Herbicide Resistance Action Committee 2022 and Fungicide Resistance Action Committee, 2022.

Pesticide	**Glyphosate**	**Azoxystrobin**	**Flutolanil**	**Fenpropimorph**	**Pencycuron**
Type	Herbicide	Fungicide			
Mode of action group	Cellular metabolism	Respiration		Sterol biosynthesis in membranes	Cytoskeleton and motor proteins
Sub-group and target site of action	Inhibition of Enolpyruvyl Shikimate Phosphate Synthase	Inhibition of complex III cytochrome bc1 (ubiquinol oxidase) at Qo site (*cyt b gene*)	Inhibition of complex II: succinate-dehydrogenase	Δ 14-Reductase and Δ 8→ Δ 7 - isomerase in sterol biosynthesis (*ergosterol 2, ergosterol 24*)	Cell division (unknown site)
Chemical group	Phosphonic acid	Methoxyacrylates	Phenyl-benzamides	Morpholines	Phenylureas
*****DT50 Soil degradation (days)	6.5	181	105	26	38
Entry mode	Systemic				Contact

* Time required for 50% dissipation under field conditions (European Commission 2024).

Azoxystrobin, flutolanil and pencycuron are fungicides that control *Rhizoctonia (Rh.) solani* in several crops e.g., sugar beet, rice or potato around the world (Buysens et al. 2015; Khan et al. 2017; Misra et al. 2023; Zhao et al. 2023). Fenpropimorph is used to control powdery mildew in wheat and to control Sigatoka diseases in bananas in exporter countries (Food and

Agriculture Organization 2017). Noticeably, glyphosate is the most widely applied pesticide in the world increasing between 1974 and 2014 from 0.0032 to 0.825 million tons per year of active ingredient applied (Benbrook 2016) and even reaching one million tons per year in 2020 (Landrigan and Belpoggi 2018).

II. 1.1.1. Azoxystrobin

Azoxystrobin is a strobilurin fungicide used worldwide (Zhang et al. 2019) against several fungal species from the Ascomycota, Basidiomycota, and Deuteromycota and fungal-like species from the Oomycota (Bartlett et al. 2002; Feng et al. 2020). It is applied on cereals, turfgrass, grapevines, potatoes and fruits, nuts, vegetables and ornamental plants (Bartlett et al. 2002). For example, commercial formulations of azoxystrobin are recommended for the control of potato diseases such as black scurf and early blight caused by *Rh. solani* [Basidiomycota] (Djébali et al. 2014) and *Alternaria solani* [Ascomycota], respectively (Landschoot et al. 2017). It is also used against *Magnaporthe oryzae* [Ascomycota] that causes rice blast, the most serious disease of cultivated rice (Kunova et al. 2014).

Azoxystrobin is principally considered as a systemic compound that belongs to the class of methoxyacrylates which exhibits its fungicidal activity by inhibition of mitochondrial respiration by binding to the quinol oxidation (Qo) site of cytochrome bc_1 complex, located in the inner mitochondrial membrane of fungi and other eukaryotes (Bartlett et al. 2002; Balba 2007; Feng et al. 2020) (**Figure 3**).

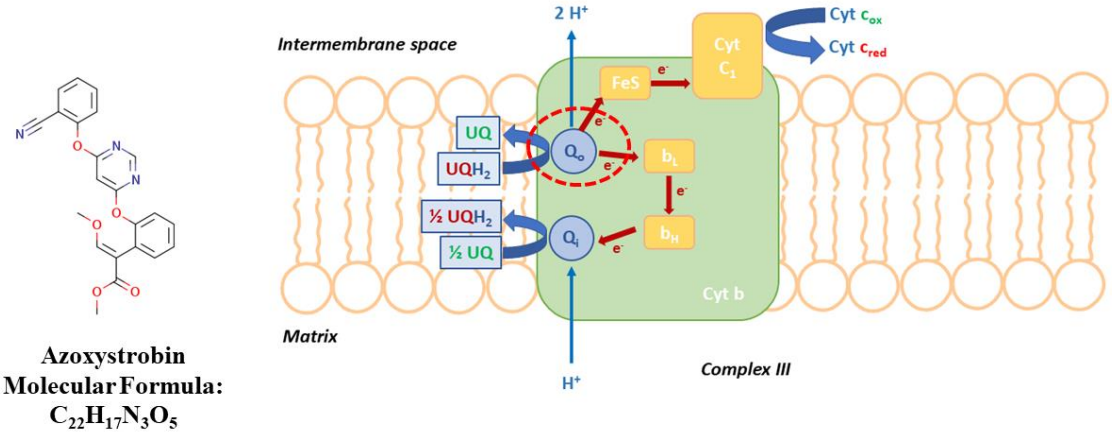


Fig. 3. Molecular formula of azoxystrobin and schematic model of cytochrome bc1 (also known as complex III) of the fungal mitochondrial respiration, with target site is the quinol oxidation (Qo) (tagged in red circle on the schema) (Musso et al. 2020).

Strobilurin fungicides have been widely used in agricultural fields for the last two decades. However, their broad-spectrum mode of action may also impact non-target soil microorganisms (Feng et al. 2020). Indeed, greenhouse studies have demonstrated that soil drenched with azoxystrobin inhibited growth of AM fungi. For example, an inhibition of root colonization was noticed in sugarcane plants inoculated with AM fungal species from the *Glomeraceae* family (Vuyyuru et al. 2018) and in maize plants inoculated with *Funneliformis coronatum* (Diedhiou et al. 2004). More recently, Alarcón et al. (2022) demonstrated, in a pot experiment, that soil application of azoxystrobin as formulation ReflectXtra (20% w/v) and isopyrazam (12.5% w/v), impacted spore density and decreased root colonization of three potato genotypes (i.e., VR808, CB2011-509 and CB2011-104) inoculated with *Claroideoglomus claroideum* and *Fulleniformis mosseae*. In contrast, foliar application of azoxystrobin did not impact root

colonization (Diedhiou et al. 2004; Hernández-Dorrego and Mestre-Parés 2010; Campos et al. 2015). A similar trend was observed with other strobilurin fungicides such as kresoxim-methyl, trifloxystrobin, pyraclostrobin applied on the leaves (Diedhiou et al. 2004) and trifloxystrobin and pyraclostrobin applied to the seeds (Cameron et al. 2007). Interestingly, strobilurin fungicides present low or very low uptake into leaves and absence of phloem mobility (Bartlett et al. 2002) probably explaining the absence of effects on AM fungi in the roots following foliar application.

Azoxystrobin is poorly soluble resulting in low bioavailability. This this strobilurin fungicide is considered persistent in soil under field conditions (European Commission 2024). Azoxystrobin was approved for use in the European Union as a fungicide on January 1, 2012, until December 31, 2024. The European Commission also approved azoxystrobin as an active substance for use in biocidal products in 2018 (European Commission 2024).

II. 1.1.2. Pencycuron

Pencycuron is a phenylurea contact fungicide, which is highly specific to *Rh. solani*, the causal agent of diseases such as rice sheath blight, potato black scurf, leaf blight of sugar beet and seedlings damping-off of various crops (Pal et al. 2005a; Young et al. 2012). It impacts the mycelial growth inducing an abnormal branching of the hyphal tips of *Rh. solani* (Yamada et al. 1988). This morphological change suggests that the mode of action of pencycuron is probably anti-microtubular (as for carbendazim), although the specific target site of pencycuron remains speculative (Fungicide Resistance Action Committee 2022). For example, whereas carbendazim inhibits β-tubulin assembly in the mitosis of *Rh. solani*, pencycuron does not have such an action but rather destroys the cytoskeleton of the microtubules (**Figure 4**) (Young et al. 2012). Several studies have suggested that the primary target site of pencycuron may be the plasma membrane (Ueyama et al. 1990; Kim and Yamaguchi 1996).

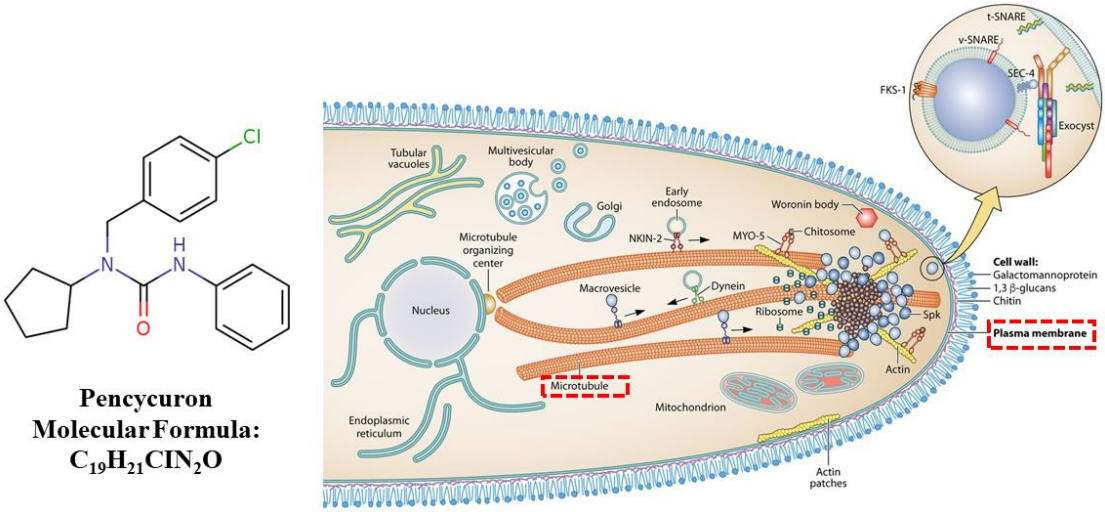


Fig. 4. Molecular formula of pencycuron and schematic representation of fungal hyphal tip with two possible target sites of pencycuron (microtubule or plasma membrane) (tagged in the red boxes on the schema) inducing an abnormal branching of mycelial growth (Riquelme et al. 2018).

Interestingly, pencycuron is the only fungicide of the phenylurea group, which also includes herbicides and plant growth regulators used in agricultural and non-agricultural systems (Navarro et al. 2012). Under laboratory conditions, this compound, applied at recommended dosage, has no effects on the soil microbial activity (Pal et al. 2005a; Wang et al. 2009).

Nonetheless, a study under greenhouse conditions demonstrated that the cytokinin-like growth regulator thidiazuron (phenylurea compounds) applied on leaves at 20 mg L^{-1} (91 µM) decreased root colonization by the non-target AM fungus *Rhizophagus intraradices* associated with the perennial grass *Miscanthus x giganteus* (Schmidt et al. 2017), suggesting an indirect effect on root colonization by a decrease in active cytokinins in the plant shoot. A modification in plant metabolism by the application of pencycuron on the leaves may thus impact AM fungal colonization more severely than a direct contact with the molecule.

Pencycuron is considered moderately persistent in soil under field conditions (European Commission 2024). However, degradation of pencycuron is often determinated for soil type. Under laboratory conditions pencycuron degradation was faster in alluvial soil (10.7 to 31.0 days) than in coastal saline soil (6.6 to 11.1 days) (Pal et al. 2005b). Organic matter amendment to soil degraded pencycuron faster than the unamended soil. Pencycuron application, although showed toxic effect on the soil microbial communities specifically fungi, the persistence was shortlived (Pal et al. 2005a). The dissipation of pencycuron under waterlogged soil field conditions ranged between 4.9 to 5.8 days. Decomposed cow manure amendment significantly accelerated the pencycuron dissipation (Pal et al. 2006).

The approval of the active substance pencycuron expired on May 31, 2021, and no application has been submitted for its renewal in the European Union. Currently, all authorisations for plant protection products containing pencycuron have been canceled (European Commission 2024).

II.1.1.3. Flutolanil

Flutolanil is a systemic phenyl benzamide fungicide, used against diseases caused by Basidiomycota in crop plants (Zhao et al. 2019). It mainly inhibits the hyphal growth and appressorium formation, and strongly reduces the mycelial O_2 consumption as well as the activity of succinate dehydrogenase (Complex II) in mitochondria (Motoba et al. 1988; Mol et al. 2019) (**Figure 5**).

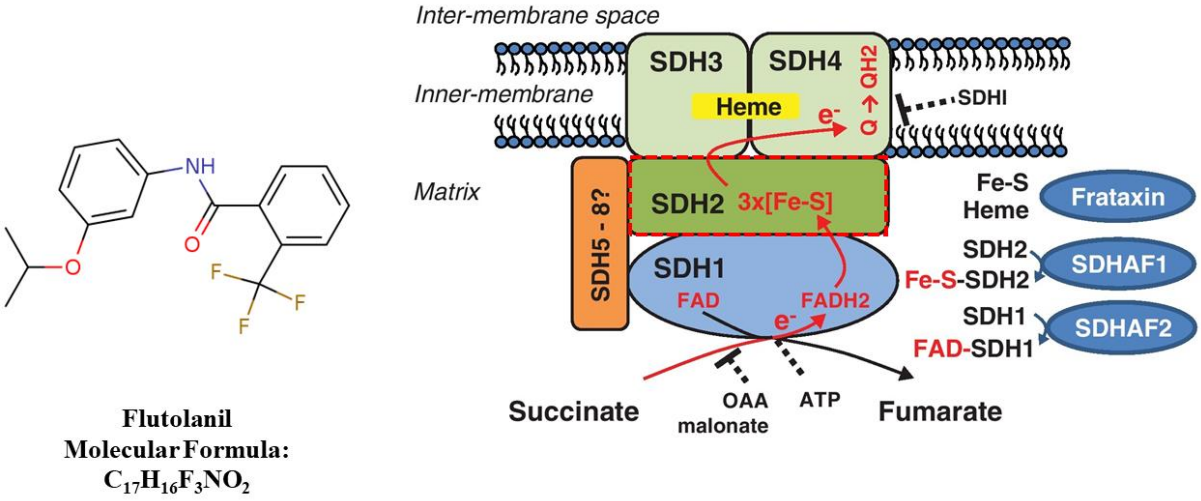


Fig. 5. The molecular formula of flutolanil and schematic representation of succinate dehydrogenase complex (also known as complex II) into mitochondria with target site of flutolanil (tagged in the red box on the schema) (Huang and Millar 2013).

Under greenhouse conditions, this compound applied on the seeds (Cameron et al. 2007) or by immersion of the root system into the fungicide solution prior to transplant (Hernández-Dorrego and Mestre-Parés 2010) did not impact AM fungal root colonization. The same observation was

made with seeds treated with sedaxane a pyrazole-4-carboxamides having similar mode of action and target site as flutolanil (Cameron et al. 2007).

Flutolanil has a low aqueous solubility, volatile with a marginal risk of leaching to groundwater (Lewis et al. 2016). Flutolanil degrades slowly in soil, primarily through microbial processes. Under field conditions this fungicide is considered persistent in soil (European Commission 2024). Flutolanil was approved for use in the European Union as a fungicide on March 1, 2009 until June 15, 2025 (European Commission 2024). The conclusions were reached on the basis of the evaluation of maximum residue levels in the representative use of flutolanil as a fungicide on potato (European Food Safety Authority 2023).

II.1.1.4. Fenpropimorph

Fenpropimorph is a morpholine of broad-spectrum activity. It is considered as a sterol biosynthesis inhibitor fungicide. It is the most important group of specific fungicides used in the world for more than three decades (Stenzel and Vors 2019). At low concentration [0.3 µM], fenpropimorph inhibits the sterol $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, and at higher concentrations [60 µM], the Δ^{14}-sterol reductase (Marcireau et al. 1990) (**Figure 6**). The interference of this compound with both enzymes leads to depletion of ergosterol and accumulation of toxic sterols, resulting in altered membrane permeability and cell death (Kerkenaar 1990; Georgopapadakou and Walsh 1996).

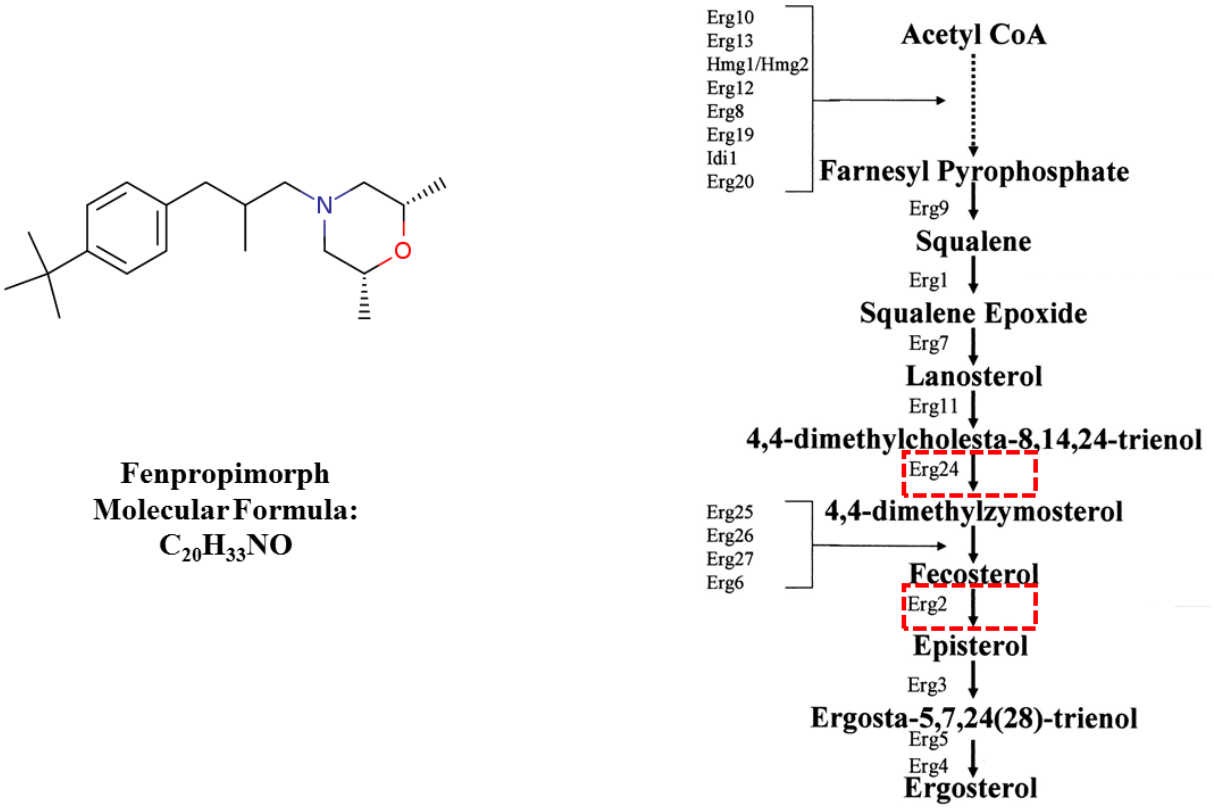


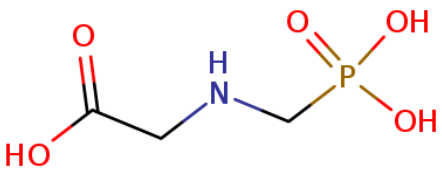
Fig. 6. Molecular formula of fenpropimorph and schematic representation of the sterol biosynthesis pathway, where fenpropimorph inhibits at low concentrations the sterol $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, and at higher concentrations, the Δ^{14}-sterol reductase (tagged in the red boxes on the schema) (Onyewu et al. 2003).

This fungicide is mainly applied in cereals to control *Blumeria* (powdery mildew) and *Puccinia* (cereal rust) fungal species (Stenzel and Vors 2019). Soil drench and foliar application of fenpropimorph have a significant impact on non-target microorganisms like AM fungi (von Alten et al. 1993; Kjøller and Rosendahl 2000). The same observation was made for other sterol biosynthesis inhibitors fungicides such as propiconazole, prochloraz, cyproconazole, myclobutanil, penconazole and fenhexamid (Nemec 1985; Plenchette and Perrin 1992; West et al. 1993; Hood et al. 2004; Hernández-Dorrego and Mestre-Parés 2010; de Novais et al. 2019a, 2019b).

Fenpropimorph has a low aqueous solubility and a low volatility (Lewis et al. 2016). The half-life of fenpropimorph in soil varies depending on the type of soil, ranging from 15 days in moderately humus loamy sand to 93 days in very humus loamy sand. Fenpropimorph is non-persistent in soil systems but may be more persistent in water systems (European Food Safety Authority 2023). Fenpropimorph was approved as an active substance in the European Union on May 1, 2009, and its approval expired on April 30, 2019 (European Commission 2024).

II.1.1.5. Glyphosate

Glyphosate is a broad-spectrum, post-emergence and non-selective chemical that kills plants by affecting the 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme of the shikimate-pathway during photosynthesis (Duke and Powles 2008) (**Figure 7**).



Glyphosate
Molecular Formula:
$C_3H_8NO_5P$

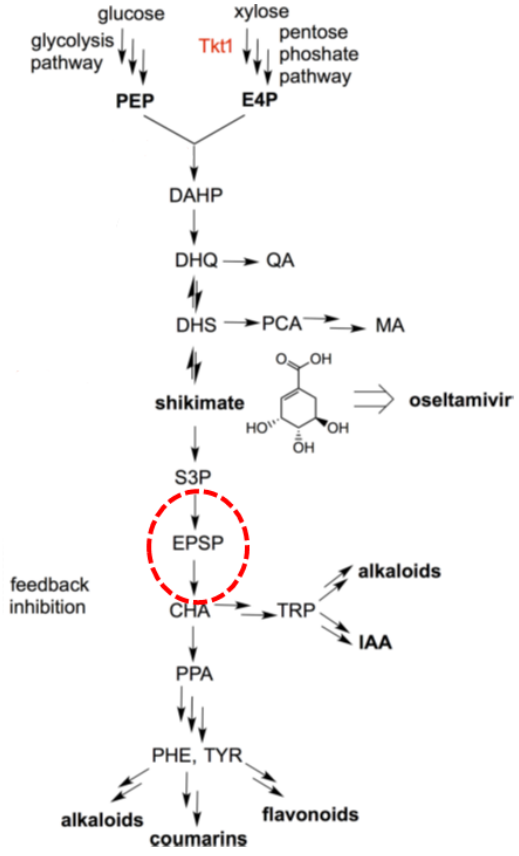


Fig. 7. Molecular formula of glyphosate and schematic representation of the biosynthetic pathways of shikimate, where glyphosate impact the 5-enolpyruvylshikimate-3-phosphate synthase (tagged in the red circle on the schema) and its derivatives metabolites (Gao et al. 2017).

Nowadays, glyphosate is the most widely used agricultural pesticide globally. Glyphosate is used extensively in genetically engineered herbicide-tolerant crops (Benbrook 2016). In addition, glyphosate is a critical compound for weed control in no-till cropping systems or conservation agriculture (Schlatter et al. 2017).

Several studies have reported contrasting effects of this compound on AM fungi. For example, under greenhouse conditions, several studies have shown no effect on AM fungi (Mujica et al. 1999; Malty et al. 2006; Powell et al. 2009; Brito et al. 2013), whereas a decrease of root colonization and spore viability was noticed in other studies (Druille et al. 2013a, 2013b, 2015; Helander et al. 2018; Wilkes et al. 2021). Field studies suggested that the high variation of results could be related to the type and dose of application, and to the interaction with other agricultural practices (e.g., plowing or fertilization) (Girvan et al. 2004; Baumgartner et al. 2005; Ronco et al. 2006; Savin et al. 2009; Pasaribu et al. 2011; Wilkes et al. 2021). Lately, under growth chamber conditions, glyphosate was shown to inhibit spore germination of *Rhizophagus clarus* but did not affect spore germination of *Gigaspora albida* (Malfatti et al. 2021). Curiously, the application of glyphosate at recommended field dose was shown to stimulate the AM hyphal length (Maltz et al. 2016) and root colonization (Reis et al. 2014) and AM fungi inoculation increased the dissipation of glyphosate in soil (Brindhavani et al. 2020).

Glyphosate is non-persistent in soil under field conditions (Bento et al. 2016). Glyphosate is primarily degraded by soil biological activity. 1) in the sarcosine pathway, glyphosate is completely mineralized via sarcosine (*N*-methylglycine) and orthophosphate due to the C-P bond cleavage; 2) in the aminomethylphosphonic acid pathway, AMPA and glyoxylate are produced via the cleavage of C-N bond of glyphosate molecules (Brock et al. 2019). Aminomethylphosphonic acid is more persistent in the environment than glyphosate (23–958 days) and tends to accumulate in the soil (Lewis et al. 2016; Bento et al. 2016). Glyphosate is currently approved as an active substance in the European Union until December 15, 2033, and its use is subject to certain conditions and restrictions. Until that date, Plant Protection Products containing glyphosate can be authorised by national authorities following a full evaluation of their safety and efficacy (European Commission 2024).

II.1.2. Pesticides impacts on the environment

The use of pesticides is the most widely spread strategy to control pests and diseases in conventional agriculture. However, it can cause substantial soil pollution, leading to potential risks to human health and ecosystems (Aktar et al. 2009; Yadav et al. 2015; Zhang Q et al. 2019; Vishwakarma et al. 2020). For instance, soil application of azoxystrobin at the maximum permitted dose of active ingredient (a.i.), i.e. 250 g ha^{-1} (1 L ha^{-1} Amistar) can lead to leaching of the a.i. and primary degradation products to aquatic environments (Jørgensen et al. 2012), impacting aquatic life (e.g., crustaceans and insects) (Giorio et al. 2021). Similarly, soil contamination with pesticides can affect non-target soil microorganisms and thus agricultural ecosystems (Riah et al. 2014; Feld et al. 2015; Zhang Q et al. 2019). They can accumulate in the soil, causing considerable changes in the ecological conditions in which the microorganisms evolve. However, the available data on the effects of pesticides on soil microflora are rather contradictory with inhibition, null or even stimulation effects reported (Prudnikova et al. 2021). For example, some bacterial taxa are sensitive to glyphosate, whereas others are not or may even benefit from it as a nutrient source such as the copiotrophic bacteria developing on dying roots (Newman et al. 2016; Schlatter et al. 2017). Earthworm activity has often been shown to decrease after application of glyphosate formulations or a.i. (Zaller et al. 2021), demonstrating the negative potential effects of this molecule on non-target essential soil organisms.

II.2. Arbuscular mycorrhizal fungi

The roots of most plants are colonized by mycorrhizal fungi which play a critical role in the capture of nutrients from soil and therefore in plant nutrition (Smith and Read 2008). These obligate root symbionts facilitate plant uptake of minerals such as phosphorus (P) and nitrogen (N) by increasing the absorbing surface area and by mobilizing sparsely available minerals (Lambers et al. 2018). In return, the host plant supplies the fungus with sugars and lipids which are essential for their life cycle (Luginbuehl et al. 2017; Wang et al. 2017). The AM fungi plays a key role in C (Parihar et al. 2020), N (Azcón-Aguilar and Barea 2015), and P (Jansa et al. 2011) cycling. They are of key interest to biologists and ecologists because they have been reported manifold to increase plant productivity and diversity, connecting plants belowground via hyphal networks allowing the movement of resources among coexisting plants (van der Heijden et al. 2015).

Four main mycorrhizal types have been described based on morphological differentiation of root tissues and host plant lineages: arbuscular mycorrhiza (AM), ectomycorrhiza, orchid mycorrhiza and ericoid mycorrhiza (Brundrett and Tedersoo 2018) (**Figure 8**).

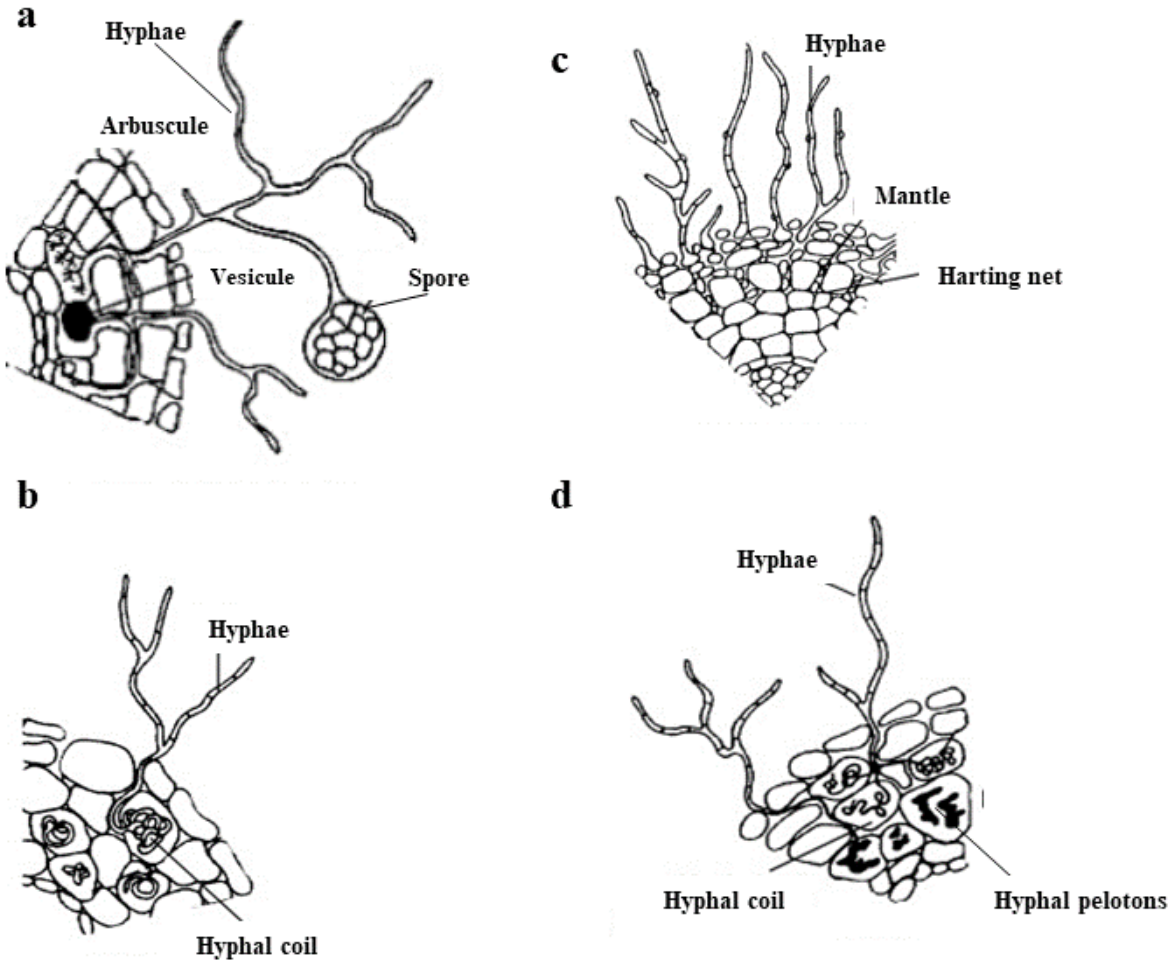


Fig. 8. Principal mycorrhizal types: **(a)** arbuscular mycorrhizal (AM) fungi (arbuscules present, vesicles present/absent and colonization from root surface mycelia or from neighboring cells); **(b)** ectomycorrhiza (Hartig net and mantle present, no intracellular colonization); **(c)** ericoid mycorrhiza (hyphal coils present, each cell is separately colonized from root surface, and mantle absent); **(d)** orchid mycorrhiza (hyphal pelotons present, colonization from root surface or from neighboring cells) (Egerton-Warburton et al. 2004; Brundrett and Tedersoo 2018).

The AM fungi belong to the Phylum Glomeromycota (Schüßler et al. 2001). They have coevolved with their host plants (Humphreys et al. 2010; Stürmer 2012) since the Devonian era, more than 450 million years ago, where they were associated with the first vascular land plants, enabling colonization of the earth surface (Rich et al. 2021). Nowadays, they are present in all terrestrial ecosystems (Stürmer and Kemmelmeier 2021), forming symbiotic associations with an estimate of 72% of vascular plants (Brundrett and Tedersoo 2018) including herbaceous and woody species, and most crop species, with a few exceptions such as the *Brassicaceae*.

II.2.1. Taxonomy and phylogeny of arbuscular mycorrhizal fungi

The AM fungi were first classified by Gerdemann and Trappe (1974) in four genera: *Glomus*, *Gigaspora*, *Acaulospora Sclerocystis*, together with the *Endogone* genera and placed in the family *Endogonaceae*, order Mucorales, phylum Zygomycota. Later AM fungi descriptions were compiled by Schenck and Peréz (1990). However, the Endogonaceae contains ectomycorrhizal fungi and non-obligatory fungi. Therefore, the Glomerales was created within the Zygomycetes to contain only the fungi being obligate biotrophs and that form arbuscules. Within this order, three families were erected: the *Glomeraceae* (*Glomus* and *Sclerocystis*), *Acaulosporaceae* (*Acaulospora* and *Entrophospora*), and *Gigasporaceae* (*Gigaspora* and *Scutellospora*) (Morton and Benny 1990). More recently, Schüßler et al. (2001) established a new phylum, the Glomeromycota, based on genetic characteristics.

The classification of Glomeromycota is still a subject of intense debate. Recently, several molecular markers (i.e. nuclear large subunit (LSU) rRNA gene, β-tubulin and mitochondrial LSU rRNA) were developed to study the evolutionary relationships of AM fungi and assess their diversity in the environment (Krüger et al. 2012). Using these molecular methods, several new AM fungi have been described, new genera erected and a strong increase in knowledge on species diversity achieved (Schüßler and Walker 2019).

Lately, a new scheme was proposed by Wijayawardene et al. (2018, 2020), with the Glomeromycota divided into three classes: *Archaeosporomycetes*, *Glomeromycetes*, and *Paraglomeromycetes* distributed in four orders (*Archaeosporales*, *Diversisporales*, *Glomerales*, and *Paraglomerales*), with 12 families and 33 genera (**Table 2**). Currently, 355 AM fungi species are validly described (Arbuscular Mycorrhizal Fungi Phylogeny, 2024).

Table 2. Classification of the phylum Glomeromycota proposed by Wijayawardene et al. (2018, 2020).

Class	Order	Family	Genera
Archaeosporomycetes	*Archaeosporales*	*Ambisporaceae*	*Ambispora*
		Archaeosporaceae	*Archaeospora*
			Palaeospora
		Geosiphonaceae	*Geosiphon*
Glomeromycetes	*Diversisporales*	*Acaulosporaceae*	*Acaulospora*
		Diversisporaceae	*Corymbiglomus*
			Desertispora
			Diversispora
			Otospora
			Redeckera
			Tricispora
		Gigasporaceae	*Cetraspora*
			Dentiscutata
			Gigaspora
			Racocetra
			Scutellospora
			Bulbospora
			Intraornatospora
			Paradentiscutata
		Pacisporaceae	*Pacispora*
		Sacculosporaceae	*Sacculospora*
	Glomerales	*Claroideoglomeraceae*	*Claroideoglomus*
		Glomeraceae	*Septoglomus*
			Sclerocystis
			Rhizophagus
			Kamienskia
			Glomus
			Funneliformis
			Dominikia
	Entrophosporales	*Entrophosporaceae*	*Eutrophospora**
Paraglomeromycetes	*Paraglomerales*	*Paraglomeraceae*	*Paraglomus*
			Innospora
		Pervetustaceae	*Pervetustus*

* Recently proposed by Błaszkowski et al. (2022).

II.2.2. The arbuscular mycorrhizal families with different life history strategies in agroecosystems

The *Glomeraceae* and *Gigasporaceae* represent the most emblematic families and along with *Acaulosporaceae* represent the most speciose families within the phylum Glomeromycota because of their distinct characteristics (Stürmer et al. 2018). *Rhizophagus irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331, selected in the present thesis, are representative species of *Glomeraceae* and *Gigasporaceae* respectively with a stable capacity to be cultivated under *in vitro* culture associated with root organ cultures. *Glomeraceae* species are dominant under conventionally tilled agricultural systems and present greater ability to colonize roots (Oehl et al. 2003; Schalamuk and Cabello 2010; Jansa et al. 2016; Xu et al. 2017; Chen et al. 2022).

Using sequencing techniques *R. irregularis* has been reported to be ubiquitous, occurring in an extremely wide range of environments, prevalent in agroecosystems irrespective field

inoculation (Peyret-Guzzon et al. 2016; Alaux et al. 2020; Renaut et el. 2020). Conversely, *Gigasporaceae* are more prevalent in stable ecosystems where inter- and intraspecific competition is high for resources, and somatic growth is favored over reproduction (de Souza et al. 2005; Lovera and Cuenca 2007; Carballar-Hernández et al. 2017; Faggioli et al. 2019). Several studies have demonstrated the high susceptibility of *Gigasporaceae* members to agricultural practices (e.g., tillage, pesticides and crop rotation) while the *Glomeraceae* appeared to be more resilient (Jansa et al. 2003; Antunes et al. 2006; Borriello et al. 2012; Rivera-Becerril et al. 2017; Soka and Ritchie 2018; Wahdan et al. 2021; Chagnon et al. 2022).

The *Glomeraceae* and *Gigasporaceae* families have developed dissimilar life history strategies (LHS) (Hart et al. 2001; Klironomos and Hart 2002). The LHS concept aims to explain how organisms invest their resources (e.g., energy) under disturbed or non-disturbed environments (IJdo et al. 2010). The *Glomeraceae* members present a high root colonization but low soil colonization, whereas *Gigasporaceae* members show the opposite trend (Hart and Reader 2002). The members of the *Glomeraceae* family allocate energy to high intraradical colonization (e.g., arbuscules, vesicles, coils and unspecialized hyphae) (de Souza et al. 2005) and abundant production of spores (r strategists[1]) (Declerck et al. 2001).

Conversely, members of the *Gigasporaceae* family invest more energy in the production of extraradical biomass than in intraradical fungal structures (Hart and Reader 2002; Maherali and Klironomos 2007). Indeed, this group is characterized by the absence of intraradical vesicles and depends mainly on the spores as a way of propagation (K strategists[2]) (Klironomos and Hart 2002; de Souza et al. 2005). They typically produce extraradical auxiliary cells which have a function in carbon storage, for use as energy for spore initiation and development and/or mycelial production and repair (de Souza and Declerck 2003).

II.2.3. The arbuscular mycorrhizal fungi life cycle

The AM fungal life cycle can be divided into (1) asymbiotic (2) presymbiotic, and (3) symbiotic phases (de Souza et al. 2005) (**Figure 9**).

[1] The r-strategists invest their resources mainly in the production of several offspring in a short time period, and evolved traits that are favored in unstable and unpredictable environments (i.e., presence of perturbation) (Begon et al. 1996).

[2] The K-strategists are organisms that evolve traits to enhance survival in stable and predictable environments (i.e., absence of perturbation) where competition for resources is high. These organisms principally allocate resources to growth and enhanced survival and presents a long-life cycle (Begon et al. 1996).

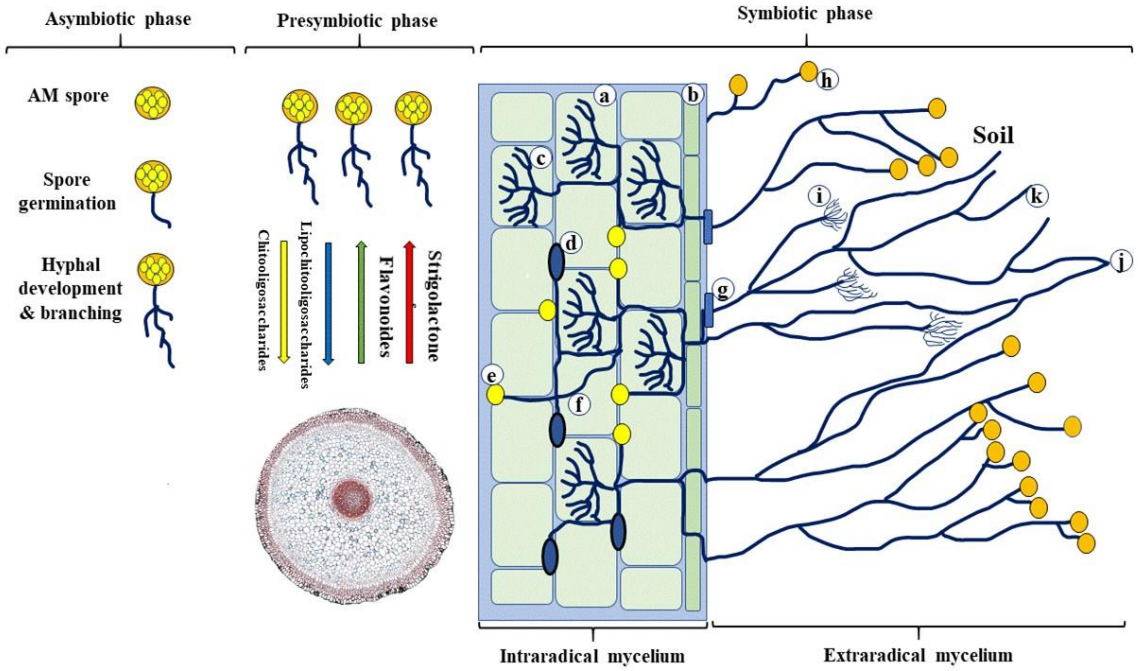


Fig. 9. Arbuscular mycorrhizal (AM) fungi life cycle divided into asymbiotic, presymbiotic and symbiotic phases: **(a)** Cortex cells in plant, **(b)** Root epidermis, **(c)** Arbuscule, **(d)** Vesicle, **(e)** Intraradical spore, **(f)** Intraradical hyphae, **(g)** Hyphopodium, **(h)** Extraradical spore, **(i)** Branched absorbing structures, **(j)** Anastomosis in the same network, **(k)** Runner hyphae. Based on Parniske 2008 and Schnepf et al. (2008).

II.2.3.1. The asymbiotic phase

The spores of AM fungi can germinate under adequate physical, chemical, and microbial conditions but present a limited hyphal growth (quiescent-like state) without the presence of a host (Giovannetti 2000). In absence of a suitable host, hyphae from a germinated spore often show retraction of protoplasm and retraction septa separating viable from empty hyphal segments (Logi et al. 1998). This process involves migration of nuclei and cellular organelles suggesting the ability of AM fungi to survive in the absence of a suitable host.

II.2.3.2. The presymbiotic phase

Root exudates stimulate spore germination, hyphal branching, and metabolic activity (Besserer et al. 2008; Bücking et al. 2008; Chang et al. 2017). Among the plant exudates that stimulate hyphal branching are the strigolactones (Akiyama et al. 2005). They activate the secretion of specific molecules in the AM fungi, the lipochitooligosaccharides called Myc factors, inducing the pre-symbiotic growth of AM fungi (Kosuta et al. 2003; Besserer et al. 2006; Rasmussen et al. 2016). Flavonoids have also been reported to stimulate AM fungal spore germination and root colonization (Pei et al. 2020; Tian et al. 2021). Upon contact with the root epidermal cells, the AM fungi form a specific anchorage structure called hyphopodium (Genre and Bonfante 2007).

II.2.3.3. The symbiotic phase

The hyphae penetrate the host epidermal cells and then the cortical cells via a pre-penetration apparatus as a guide (Genre et al. 2005). Ultimately, it forms a highly branched tree-like structure called arbuscule. This structure is surrounded by a peri-arbuscular membrane which

separates it from the cytoplasm of the host plant (**Figure 10**) (Parihar et al. 2020). The arbuscule is intimately connected to the cortical cell and is considered the central functional unit of symbiosis as it mediates the bi-directional nutrient exchange between the two partners of symbiosis (Pimprikar and Gutjahr 2018).

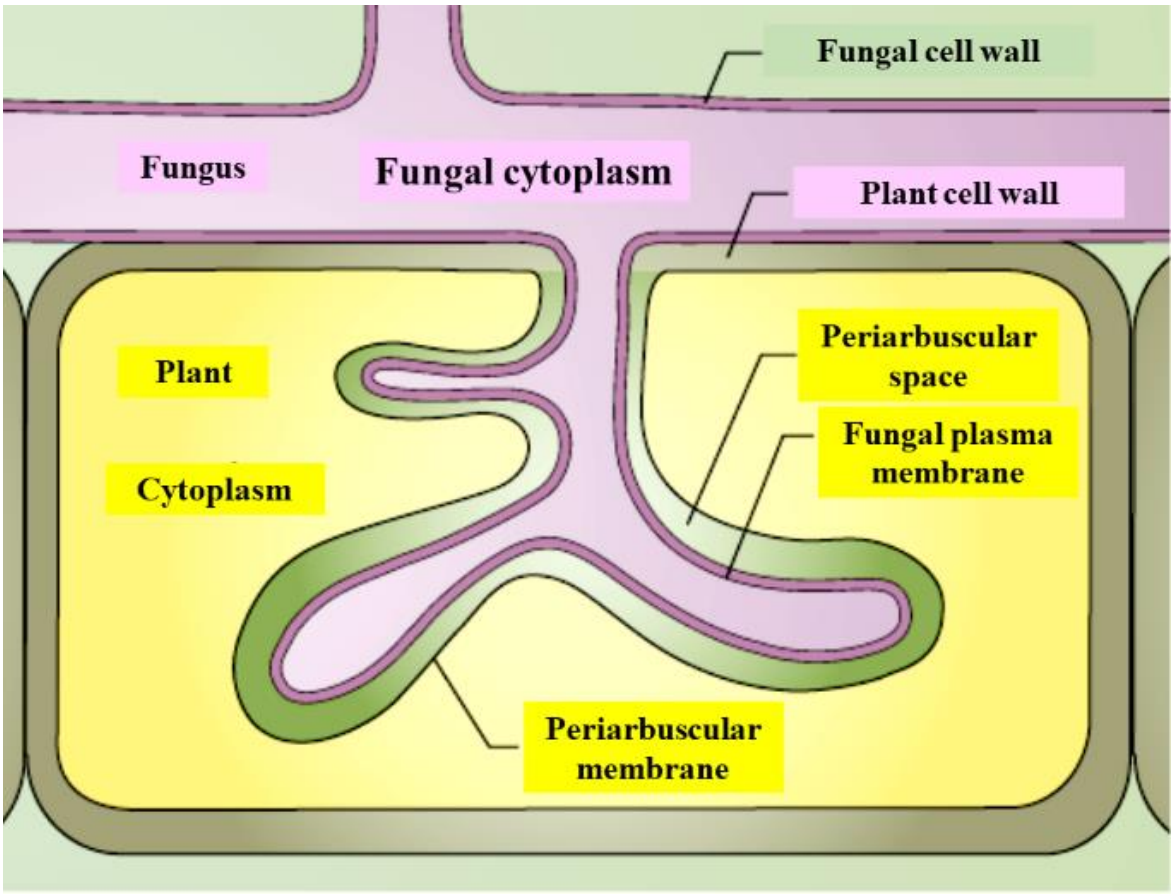


Fig. 10. Schematic representation of an arbuscule within a cortical cell. The hyphae of the AM fungus invaginate the plasma membrane to increase the surface of exchange between the fungal and plant cell, both are separated by a peri-arbuscular space (Parniske 2008).

The arbuscules can have two different morphologies: Arum or Paris (Smith and Smith 1997). In the Arum-type, the fungal hyphae grow longitudinally in the intercellular spaces between the cortical cells and penetrate the cells to differentiate into arbuscules (Smith and Read 2008). Conversely, in the Paris-type, the hyphae grow directly from cell to cell so that the intercellular phase of development is highly restricted or completely absent. Complex coils develop within colonized cells of Paris-type, which may bear arbuscule-like branches (arbusculate coils) (Bonfante and Genre 2008). In general, the Arum-type is more common in crop plants, while the Paris-types is preferentially found in plants growing in natural ecosystems (Ahulu et al. 2005). At the final step of root colonization, the members of all families, except for the *Gigasporaceae*, develop vesicles in the cortex (De Souza et al. 2005). The vesicles are thick-walled structures of varying shapes from ovoid, irregularly lobed to box-like, depending on the fungal species. They contain abundant storage lipid bodies and numerous nuclei and it is likely that they are important storage organs, playing a significant role as propagules within root fragments (Smith and Read 2008).

Following the development of the profuse intraradical mycelium, the AM fungus starts to proliferate into the soil as thick-walled hyphae called runner hyphae (Friese and Allen 1991;

Bago et al. 1998). These runner hyphae can extend rapidly, colonizing the soil and connecting new roots (Dalpé et al. 2005), belonging to the same or different plant taxa (Pepe et al. 2018). The ERM also develops branched absorbing structures which are involved in the uptake of minerals (de Souza and Declerck 2003). During symbiosis, spores, and auxiliary cells (in the case of *Gigasporaceae*) are formed on the ERM (Smith and Read 2008). Spores are formed apically or intercalary along the runner hyphae of higher order and represent the final stage in the fungal life cycle (Declerck et al. 2004). Recently, it has been shown that the ERM represents a long-term survival structure, remaining functional at least 5 months after host roots death (Pepe et al. 2018). Interestingly, the ERM can make anastomosis (see II.2.4.5.), a process to interconnect different fungal networks.

II.2.3.5. Anastomosis in arbuscular mycorrhizal fungi with different life history strategies

Anastomosis is the "*process of fusion between branches of the same or different hyphae to produce a mycelial network that involves degradation of the cell wall, followed by plasma membrane fusion*" (Glass and Fleissner 2006). This mechanism fulfills several functions such as heterokaryon formation, intra-hyphal communication, and homeostasis maintenance (Glass et al. 2000). In AM fungi, the hyphal anastomosis allows the physical exploration of the environment by forming a three-dimensional network, favoring protoplasmic/cytoplasmic flow and genetic material exchange (Giovannetti et al. 2001; Croll et al. 2009; Daubois et al. 2016; de Novais et al. 2017) (**Figure 11**). Indeed, interconnectedness of mycorrhizal networks is the result of anastomoses (Giovannetti et al. 2015).

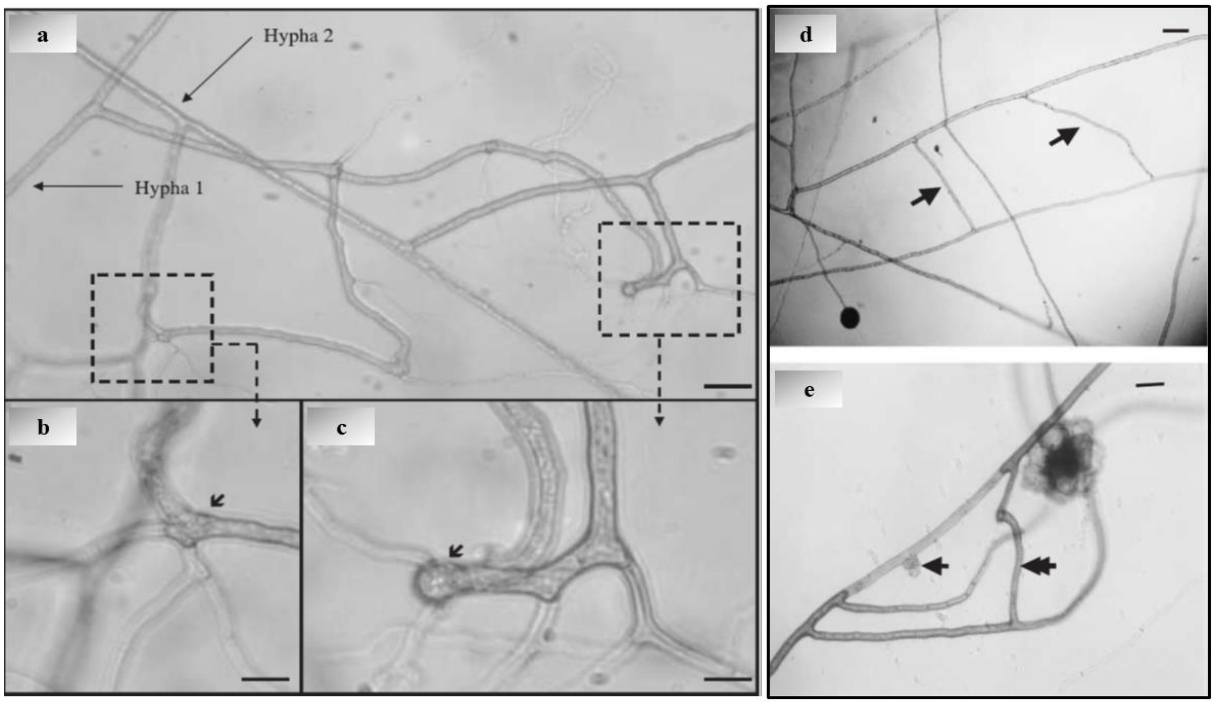


Fig. 11. Anastomosis formation between two hyphae of different arbuscular mycorrhizal (AM) fungal networks of the same isolate of *Rhizophagus prolifer* **(a)** two anastomoses are observed (square boxes). Bar, 50 μm **(b, c)** detailed view of the anastomoses, formed by a tip-to-tip connection (arrows). Bars, 20 μm (Voets et al. 2006). Anastomosis in intact mycelial networks **(d)** between two primary hyphae of *Rhizophagus intraradices* (arrows). Bar, 100 μm and **(e)** within the same hyphae (hyphal bridge) of *Scutellospora reticulata* following cytoplasmic/protoplasmic flow interruption (double arrow). Bar, 40 μm (de la Providencia et al. 2005).

Anastomosis formation in AM fungi has been described and quantified in hyphae derived from germinated spores and symbiotic mycelial network (De Souza and Declerck 2013; Purin and Morton 2013). It is to be noticed that the interaction among mycelia produced by different isolates or lineages may also lead to pre-fusion incompatibility which hinders anastomosis formation, or to incompatibility after fusion, which separates the hyphal compartments (de Novais et al. 2017; Sbrana et al. 2018).

Interestingly, AM fungal species belonging to the *Glomeraceae* and *Gigasporaceae* present different patterns and densities of anastomosis (Giovannetti et al. 1999; de Souza and Declerck 2003; de la Providencia et al. 2005; Voets et al. 2006; Schnepf et al. 2008; Purin and Morton 2011; de Novais et al. 2013). Both families have thus developed different strategies to survive and colonize the plants under adverse conditions (de la Providencia et al. 2007). It has been shown that independent hyphal networks belonging to the same isolate in the *Glomeraceae* were able to interconnect, while in the *Gigasporaceae* such process was rarely observed (Giovannetti et al. 1999; Voets et al. 2006; Purin and Morton 2011). Conversely, in *Glomeraceae* members, ERM networks are highly interconnected by means of anastomoses whose frequency was positively correlated with plant growth and P nutrition (Avio et al. 2006). Notably, the anastomosis is regulated evolutionarily following a balance between negative (e.g., virus transmission and aggressive nuclei transfer) and positive functional consequences (e.g., increasing genotypic diversity and plasticity and favoring response to disturbance) (Chagnon 2014).

The hyphae of soil fungi are susceptible to damage by factors such as grazing by mites and other arthropods and by osmotic rupture through sudden changes in external water potential (Carlile 1995). By breaking up soil, tillage often disrupts mycelial networks in agricultural systems (Wu et al. 2016; Cruz-Ortiz et al. 2020). Indeed, anastomosis indistinctly concerns intact and damaged hyphae, the latter being described as a hyphal healing mechanism (HHM). The first description of wound-healing in AM fungi after physical injury of hyphae was reported with spores cultivated on hemp seed agar (Gerdemann 1955). Later, HHM was reported in *Gigasporaceae* members (e.g., *Gigaspora margarita*, *Scutellospora verrucosa* and *S. heterogama*) with spores cultivated on agar media (Bécard and Piché 1989; Kang-Hyeon et al. 1994). The term ‘hyphal healing’ was suggested as a mechanism of hyphal survival linked to ageing and/or lytic events of ERM (Bago et al. 1999).

Finally, the HHM in symbiotic phase has been described in detail under *in vitro* conditions in four successive events by de la Providencia et al. (2005). First, it begins with the formation of a septum near or in the apical zone on both sides of the injured hyphae, which prevents massive cytoplasmic/protoplasmic leakage into the surrounding environment. Secondly, one or several growing hyphal tips (GHTs) emerge from both extremities of the cut hyphae either protruding through the septum or emerging behind it. Thirdly, the GHTs elongate and grow towards each other and in most cases enter into contact. Fourthly, fusion is observed between GHTs with re-establishment of cytoplasmic/protoplasmic flow (de la Providencia et al. 2005) (**Figure 12**).

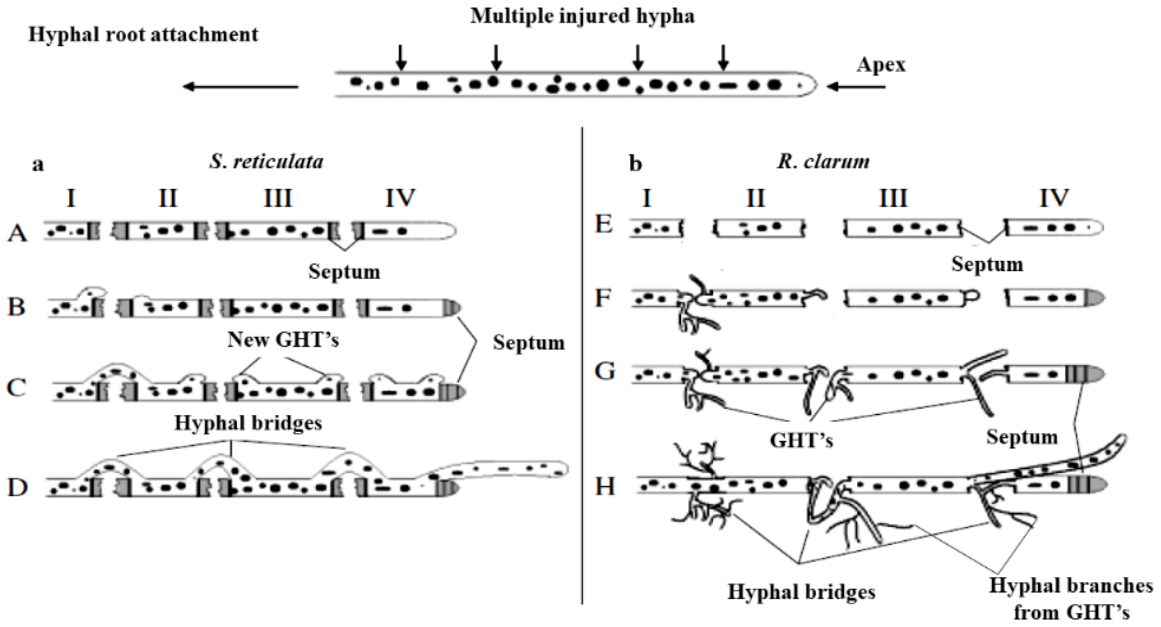


Fig. 12. Schematic representation of the hyphal healing mechanism (HHM) in multiple-injured hyphae of *Scutellospora reticulata* and *Rhizophagus clarum*. In *S. reticulata*, **(A)** septa are produced at the extremities of each cut section **(B)** the first GHT appears behind the septum in section I. **(C)** other GHTs appear behind the septa of the other sections. In section IV, multiple septa are formed and a GHT appears at the apex side, behind the last septum. The GHTs of sections I and II always fuse before the other sections. **(D)** the healing proceeds further by connecting all the sections. After total reconnection of the sections, the GHT at the tip of the apex section becomes the main apex and starts its elongation and exploration of the substrate. In *R. clarum*, **(E)** septa are produced at the cut-extremities of each section. **(F)** the first GHT initiates its elongation throughout the septum but does not always correspond to section I. **(G)** several GHTs are produced within the hyphal sections and also multiples septa are formed in the apex side of section IV but no GHT is noted at this side. **(H)** Reconnection of all the sections (de la Providencia et al. 2007).

Importantly, HHM is considered a major process allowing the AM fungi to maintain mycelial integrity following physical injury (Garcés-Ruiz et al. 2021). Curiously, the HHM differ between *Glomeraceae* and *Gigasporaceae* members (de la Providencia et al. 2005, 2007) (**Figure 13**). For instance, the HHM in *Glomeraceae* (e.g., *R. irregularis*, *R. prolifer*, and *R. clarus*), is oriented towards the reconnection of the affected area by linking several hyphae in relatively small vicinity or by recolonization of roots and substrate, by contrast to *Gigasporaceae* (e.g., *S. reticulata*, *G. margarita*, *G. rosea*) in which the HHM is nearly always oriented towards the re-establishment of hyphal integrity (de Souza and Declerck 2003; de la Providencia et al. 2007).

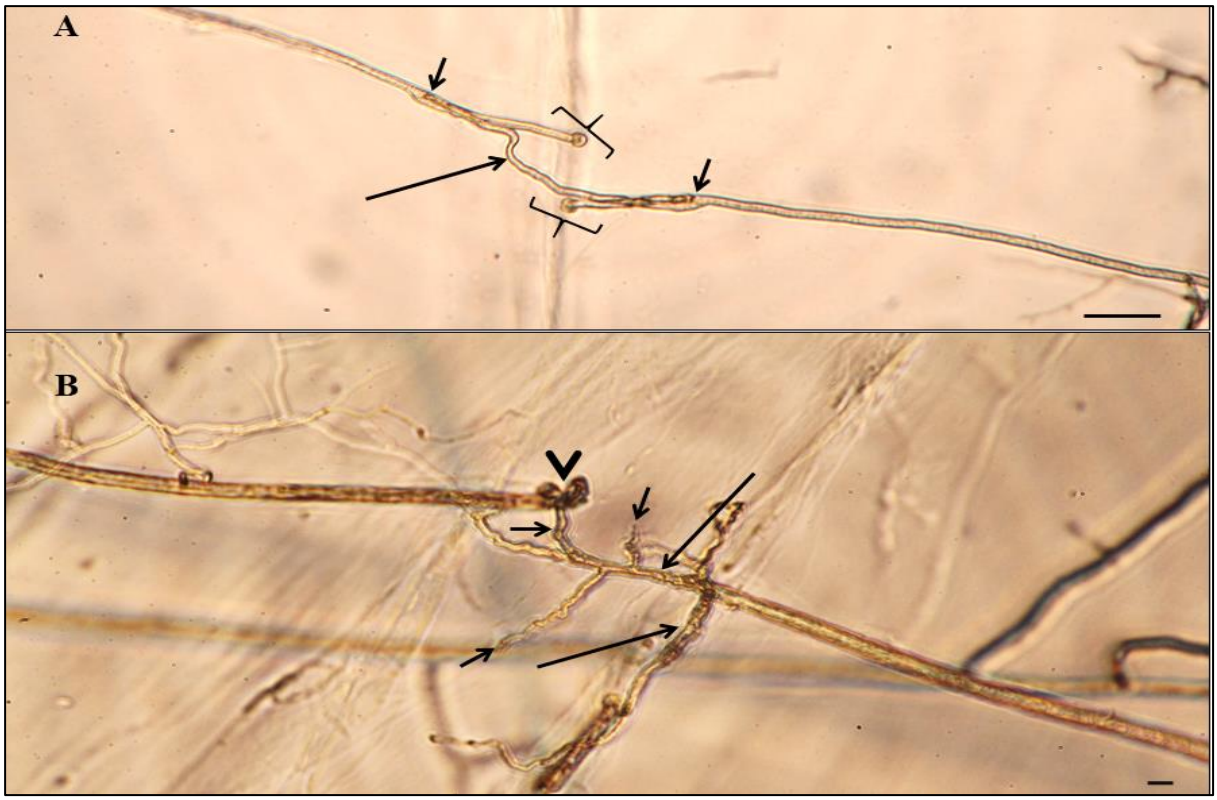


Fig. 13. The hyphal healing mechanism (HHM) of arbuscular mycorrhizal (AM) fungi **(A)** in *Gigaspora* sp. MUCL 52331, plugs are formed at both extremities. In front of the hyphal injury (braces), two GHTs emerge (large arrows) at both extremities behind the septum (short arrows). After GHT contact and fusion, cytoplasmic/protoplasmic flow is re-established (scale bar 50 μm) **(B)** in *Rhizophagus irregularis* MUCL 41833, the plugs are formed at both extremities in front of the hyphal injury (braces) and three GHTs emerge through the extremities of the hyphal injury (large arrows). After GHT contact and fusion (arrowhead), cytoplasmic/protoplasmic flow is re-established. Several hyphal branches extending from GHTs (short arrows) are observed (scale bar 10 μm) (pictures from M Calonne-Salmon, laboratory of Mycology of UCLouvain).

Environmental factors (e.g., soil pollution) may influence HHM integrity and functions. For instance, hydrocarbon pollutants such as diesel was shown to differentially affect the HHM of members belonging to the two above-mentioned families of AM fungi (Garcés-Ruiz et al. 2021). For example, all steps of HHM in *R. irregularis* were severely impaired. Conversely, the HHM in *Gigaspora* sp. was more tolerant to pollutant exposure. These results support that both fungi belonging to phylogenetically distant families have different responses to abiotic stresses.

II.3. Benefits of the symbiosis for the host plants

The AM fungi are dependent on carbon (C) and fatty acids supplied by the host plant to accomplish their life cycle. In exchange, they provide the plant with mineral nutrients scavenged from the soil by their ERM, resulting in increasing plant growth (Parniske 2008; Grace et al. 2009). This increase of plant biomass is often expressed as mycorrhizal responsiveness (Lehmann et al. 2012). The mycorrhizal responsiveness in agricultural fields is regulated by several factors such as P availability, plant species, and AM fungal identity (Li et al. 2005; et al. 2013; Peña-Venegas et al. 2021; Chu et al. 2020; Zhang et al. 2021).

Remarkably, phosphate delivery is among the most important benefits for the host plant in AM symbiosis (Li et al. 2006; Wang et al. 2017). Indeed, P is a major nutrient, limiting crop production in many regions of the world. Because of the capacity of AM fungi to improve plant P acquisition, there is much interest in applying AM fungi in agriculture to increase crop yields while simultaneously reducing the amount of P fertilizer applied to crops (Hijri 2016; Peña-Venegas et al. 2021).

The root systems of main consumed crops in the world are colonized by diverse assemblages of AM fungi (Verbruggen and Kiers 2010). The AM root colonization produces potential functional benefits (e.g. nutrient transfer, pathogen protection, water uptake) in the crops. Although, the beneficial effects of AM symbiosis in field may be regulated by competition with local organisms, to agricultural practices and to edapho-climatic conditions (Liu et al. 2016; Wilkes et al. 2021).

Interestingly, intact ERM colonizes roots faster than spores, colonized root segments, or fragmented hyphae. This is especially important when crops are exposed to soil stresses, as early colonization can help the plant get the most benefit during the symbiosis (Goss et al. 2017; Brito et al. 2021). In general, several studies have reported that ERM can survive in agricultural soils with a permanent or semi-permanent organic cover on the soil, varied crop rotation and minimum tillage or no-till (Hijri et al. 2006; Brito et al. 2011, 2012, 2019, 2021; Brígido et al. 2017; Edlinger et al. 2022).

II.3.1. Transport of mineral elements

Studies on crop plants including cereals, vegetables and fruit trees inoculated with AM fungi have shown increased leaf area, and nitrogen, potassium, calcium, and phosphorus contents, resulting in enhanced plant growth and yield (Balliu et al. 2015; Lone et al. 2015; Chen et al., 2018; Shahvali et al. 2020).

Notably, plants preferentially absorb P as inorganic phosphate (Pi), a limiting ion in many environments due to its low solubility and mobility in soil. Remarkably, AM fungi can ameliorate P nutrition of plants by accessing remote locations of Pi through their ERM (Johri et al. 2015; Ferrol et al. 2019). This improved Pi nutrition can maintain optimal growth and therefore increase the tolerance of plants to biotic (Diagne et al. 2020) and abiotic stresses (Lenoir et al. 2016; Begum et al. 2019).

Mycorrhizal plants have two pathways for taking up Pi from the soil solution. Direct Pi uptake occurs via the root epidermis and root hairs, whereas in the AM fungal pathway, Pi is taken up by external AM hyphae and transported to the intracellular symbiotic interfaces within colonized root cortical cells (Grace et al. 2009). The transfer of Pi from the AM fungus to the plant involves high affinity H^+: Pi transporters (Harrison and van Buuren 1995; Plassard et al. 2019). AM fungi induce the expression of phosphate transporters. Symbiotic Pi uptake by phosphate transporters is crucial for mycorrizal colonization and arbuscule dynamics (Liu et al. 2018). These transporters are localized to the plant periarbuscular membrane for Pi uptake (Banasiak et al. 2021). For example, expression studies performed with different tissues of mycorrhizal potato plants allowed the identification of AM fungi inducible plant phosphate transporters (i.e. StPT3, StPT4), which are involved in the transfer of the uptake of fungus-delivered phosphate in colonized plant cells (Rausch et al. 2001; Karandashov et al. 2004; Nagy et al. 2005).

On the other hand, Pi transporters of AM fungi have been identified: *R. irregularis* (GiPT), *Funneliformis mosseae* (GmosPT), *R. clarus* (GvPT), and *Gigaspora margarita* (GigmPT). They all share structural and sequential similarities with other plant and fungal high affinity with phosphate transporters (Karandashov and Bucher 2005; Rui et al. 2022). GiPT transcript was detected mainly in extraradical mycelium and gene expression was correlated with the external Pi concentration, indicating their role in acquiring Pi from the soil (Maldonado-Mendoza et al. 2001). AM fungi depolymerize inorganic polyphosphate (poly P) before providing Pi to the host (Ohtomo and Saito 2005). Following translocation from the extraradical mycelium to the intraradical mycelium, poly-P is thought to be hydrolyzed by phosphatases (Solaiman et al. 1999). Hydrolysis of poly-P at sites of P utilization or transfer to the plant probably occurs through the combined activities of exopolyphosphatases, endopolyphosphatases and non-specific acid phosphatases (Müller et al. 2019). Alkaline phosphatase and acid phosphatases activities have also been detected in extra- and intraradical hyphae (van Aarle et al. 2001; Funamoto et al. 2007). The final steps of the process require the release of Pi into the peri-arbuscular apoplastic compartment and the transfer to plant cells via membrane transporters (Smith and Smith 1990). Mostly, acid and alkaline phosphatases catalyze the hydrolysis of several organic phosphate-monoesters yielding the soluble P required for growth. Mainly, alkaline phosphatase activity has been used to measure microbial activity in the soil or efficiency of AM fungi on plant nutrition and has been proposed as an indicator of the proportion of AM hyphae involved in P metabolism during AM symbiosis (Tisserant et al. 1993; Guillemin et al. 1995).

The contribution of AM fungi to P uptake by plants can be identified by comparison with P uptake in plants in absence of these symbionts (Li et al. 2006). Indeed, it is well known that mycorrhizal plants can take up and accumulate more Pi than non-mycorrhized plants under normal growth conditions (Garcés-Ruiz et al. 2017) and even under abiotic stress (e.g., pollution, drought) (Calonne-Salmon et al. 2018; Le Pioufle et al. 2019) conditions.

II.3.2. Protection against abiotic stress as saline, drought, and pollution

Abiotic stresses, such as drought, salinity, extreme temperatures, and exposure to pollutants result in the deterioration of the soil and present serious threats to agriculture because they are considered as the primary cause of crop yield loss worldwide (Wang et al. 2003). Nowadays, climate change and inadequate agricultural practices like excessive use of fertilizers and pesticides have aggravated the effects of abiotic stresses on crop productivity (Begum et al., 2019). The potential of AM fungi to increase plant resistance/tolerance to abiotic stress conditions is largely documented (Wu et al. 2013; Plouznikoff et al. 2016; Begum et al. 2019). However, knowledge of the mechanisms implemented by AM fungi themselves to tolerate the deleterious effects induced by stresses is still limited (Lenoir et al. 2016).

II.3.3. Impact of arbuscular mycorrhizal fungi in potato yield

Potato (*Solanum tuberosum*), with a global production of 375 Mt in 2022 (Food and Agriculture Organization 2024), is the world's fourth largest food crop, following maize, rice, and wheat. It is considered critical for food security in a world confronted by increasing population growth (Devaux et al. 2021). Potato is a high phosphorus-demanding crop (Wacker-Fester et al. 2019) usually is simultaneously colonized by diverse AM fungal species (e.g., *R. irregularis*) in field irrespective field inoculation (Senés-Guerrero et al. 2014; Buysens et al. 2017). Understanding and managing its AM association is of agricultural interest. For instance, recent studies confirmed positive responses on grow and tuber biomass of *S. tuberosum* have after inoculation

with *R. irregularis* in pot experiments (Yang et al. 2020; Deja-Sikora et al. 2023; Terry et al. 2023). Interestingly, inoculation with AM fungi in potato crop is a promising option to increase tuber yield. However, in most cases, the inoculated strains could not be traced in the field and their contribution to root colonization separated from native AM fungi (Senés-Guerrero et al. 2014; Buysens et al. 2017). Results of large-scale field trials indicated that inoculation of potato with *R. irregularis* caused significant increase in tuber yield, but the effect was cultivar independent under low fertilization treatments (Hijri, 2016; Boussageon et al. 2023). Indeed, using pot experiments authors demonstrate that fungicide application and AM fungi incoculation impacts tuber yield diferencially according to potato genotype with different phenolic compounds and antioxidant activity in tubers (Alarcón et al. 2022; Fritz et al. 2022).

Furthermore, the root colonization of potato with AM fungi is often linked to a lower incidence of infection with some pathogens or reduced disease severity. For example, *C. etunicatum* and *R. irregularis* were associated with milder symptoms of *Rh. solani* in potato (Yao et al. 2002). In addition, AM fungi have been shown to decrease leaf symptoms caused by *Phytophthora infestans* in controlled and field conditions, because of the activation of plant systemic resistance to this pathogen (Gallou et al. 2011, Alaux et al. 2018). More recently, results obtained under field conditions suggested that potato virus Y (PVY) infection, which is one of the most important potato diseases, can be mitigated by mycorrhizal symbiosis, which reduces the oxidative stress in PVY-infected host plants (Deja-Sikora et al. 2020). In another study conducted under *in vitro* culture conditions, Buysens et al. (2015) suggested that fungicides at recommended field dosage to control *Rh. solani* may be combined with AM fungi. In contrast, fungicides at higher concentrations as azoxystrobin may inhibit growth and benefits of AM symbiosis.

II.4. Impact of pesticides on the arbuscular mycorrhizal symbiosis

II.4.1. Impact of pesticides on arbuscular mycorrhizal fungal diversity in soils

In agricultural systems, conventional management practices such as the use of chemical pesticides influence AM fungal species composition (Kurle and Pfleger 1996; Homma et al., 2012; Bedini et al. 2013; Rivera-Becerril et al. 2017; Carballar-Hernández et al. 2017). For example, the continuous application of fungicides has been reported to decrease the AM fungal diversity in agricultural soils (Henning et al. 2018; Ren et al. 2021). This impact was confirmed in combination with other practices such as the use of inorganic fertilizers (Bordoloi et al. 2015) or ploughing (Castelli et al. 2014). Alteration in AM fungal species composition following fungicide application has also been reported (Land et al. 1993; Carrenho et al. 1998). For instance, Helgason et al. (2007) and Schalamuk et al. (2014) demonstrated that in presence of the fungicides benomyl and trifloxistrobin-tebuconazole, the number of *Glomeraceae* species increased, while the number of *Acaulosporaceae* species decreased and those of *Gigasporaceae* remained unaffected.

Herbicides may differentially affect AM fungal community composition and abundance in soil (Wang et al. 2012; Alguacil et al. 2014; Chikoye et al. 2014; Melloni et al. 2017; Parsons et al. 2020; Nogales et al. 2021). For instance, diversity of AM fungi in field was not affected by nicosulfuron, a systemic selective herbicide (Karpouzas et al. 2014; de Freitas et al. 2018). Likewise, null effect was observed with post-emergence applications of glyphosate and oxyfluorfen or pre-emergence applications of oxyfluorfen and simazine (Baumgartner et al. 2010). Conversely, AM fungal diversity was reduced after field application of imazaquin (0.10, 0.20 and 0.40 kg a.i. ha^{-1}) and pendimethalin at 8 kg a.i. ha^{-1}. A similar observation was made

in the field after application of oxyfluorfen at 1.5 kg ha^{-1} (Alguacil et al. 2014). A detrimental impact in AM fungal diversity was noticed by nicosulfuron application although only above the recommended field dosage (x100 and x1000) in a pot experiment (Karpouzas et al. 2014). In addition, pre-emergence application with glyphosate, oxyfluorfen and simazine at 0.62, 1.38 and 0.92 kg a.i. ha^{-1} decreased AM fungal spore populations in the field (Baumgartner et al. 2005).

Finally, studies focusing on the impact of insecticides on AM fungi diversity are rare. Curiously, soil application of chlorpyrifos, a broad spectrum and organophosphate insecticide, increased AM fungal diversity associated with grass species (Vandenkoornhuyse et al. 2003). Although, these authors suggested an indirect effect by lower predation pressure related to a depletion of mesofaunal populations, especially mycophagous collembolan species.

II.4.2. Impact of fungicides and herbicides on the development of arbuscular mycorrhizal fungi with different life history strategies in greenhouse and field conditions

As mentioned above, pesticides also affect soil plant-beneficial microorganisms such as AM fungi. They are exposed to pesticides applied as soil drench, seed treatment or even foliar spray for systemic compounds or compounds present in run-off from leaf application (Hage-Ahmed et al. 2019). Due to their key role in soil fertility, AM fungi have been proposed as important non-target microorganisms to be monitored in order to evaluate the environmental impact of pesticides (Wan et al. 1998; de Novais et al. 2019a). Indeed, they have been listed by the European Food Safety Authority as organisms to be included in the test battery for the risk assessment of plant protection product (European Food Safety Authority 2017; Sweeney et al. 2022).

After herbicides, fungicides are the most used pesticides in conventional agriculture. Several studies have been conducted under greenhouse and field conditions using mainly foliar and soil applications to determine the impact of different fungicides (in addition to benomyl, a fungicide used widely to eliminate AM fungi in experiments) on AM fungi, considering mainly the percentage of roots colonization and production of spores (**Table 3**).

Table 3. Description and effects of fungicides having different modes of action on arbuscular mycorrhizal fungi.

Fungicide	Concentration	AM fungi	Host plant	Culture conditions	Principal effects on AM fungi	Reference
C: Respiration						
C2 complex II: succinate-dehydrogenase						
Flutolanil	1.2 g L^{-1}	*R. intraradices* *F. mosseae*	*Allium porrum*	Greenhouse/root immersion	Null effect on RC	Hernández-Dorrego and Mestre-Parés 2010
C3 complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene)						
Azoxystrobin	50, 500, 5000 mg L^{-1}; 50 mg kg^{-1}	*F. coronatum*	*Zea mays*	Greenhouse/soil drench or foliar application	Null effect on RC and EA (foliar application). Inhibition of RC and EA by soil drench at increasing doses.	Diedhiou et al. 2004
	1.5 g L^{-1}	*R. intraradices* *F. mosseae*	*Allium porrum*	Greenhouse/ root immersion, soil or foliar application	Null effect on RC (foliar application). Inhibition of RC (Soil drench).	Hernández-Dorrego and Mestre-Parés, 2010
	120 g ha^{-1}	Indigenous AM fungi	*Phaseolus vulgaris*	Greenhouse/foliar application	Null effect on RC and SP	Campos et al. 2015
	296 kg g ha^{-1}		*Saccharum* spp.	Greenhouse/soil drenched	Inhibition of RC	Vuyyuru et al. 2018
G: Sterol biosynthesis in membranes						
G1 C14- demethylase in sterol biosynthesis						
G2 sterol $\Delta^8 \rightarrow \Delta^7$-sterol isomerase						
Fenpropimorph	1875 ppm	*R. intraradices* *F. mosseae*	*Hordeum vulgare*	Greenhouse/foliar application	Inhibition of RC	von Alten et al. 1993
	1.25, 125 µg g^{-1}	*F. caledonius*	*Cucumis sativus*	Greenhouse/soil drench	Inhibition of EA activity of ERM	Kjøller and Rosendahl 2000

Abbreviations: RC: Root colonization, **SP:** Spore production, **SG:** Spore germination, **EA:** Enzymatic activity, **HL:** hyphal length, **NA:** Not applicable.

Over fifty percent of the studies have reported an inhibitory effect with contact or systemic fungicides (**Figure 14**). Neutral or beneficial effects have also been reported whatever the mode of entry of the fungicides. Currently, the different effects of systemic and contact fungicides on the AM fungi are not fully understood. These interactions deserve attention because AM fungi can help plants fend off pests and diseases, thus potentially reducing/optimizing fungicide application strategies (Yang et al. 2011; Santísima-Trinidad et al. 2018).

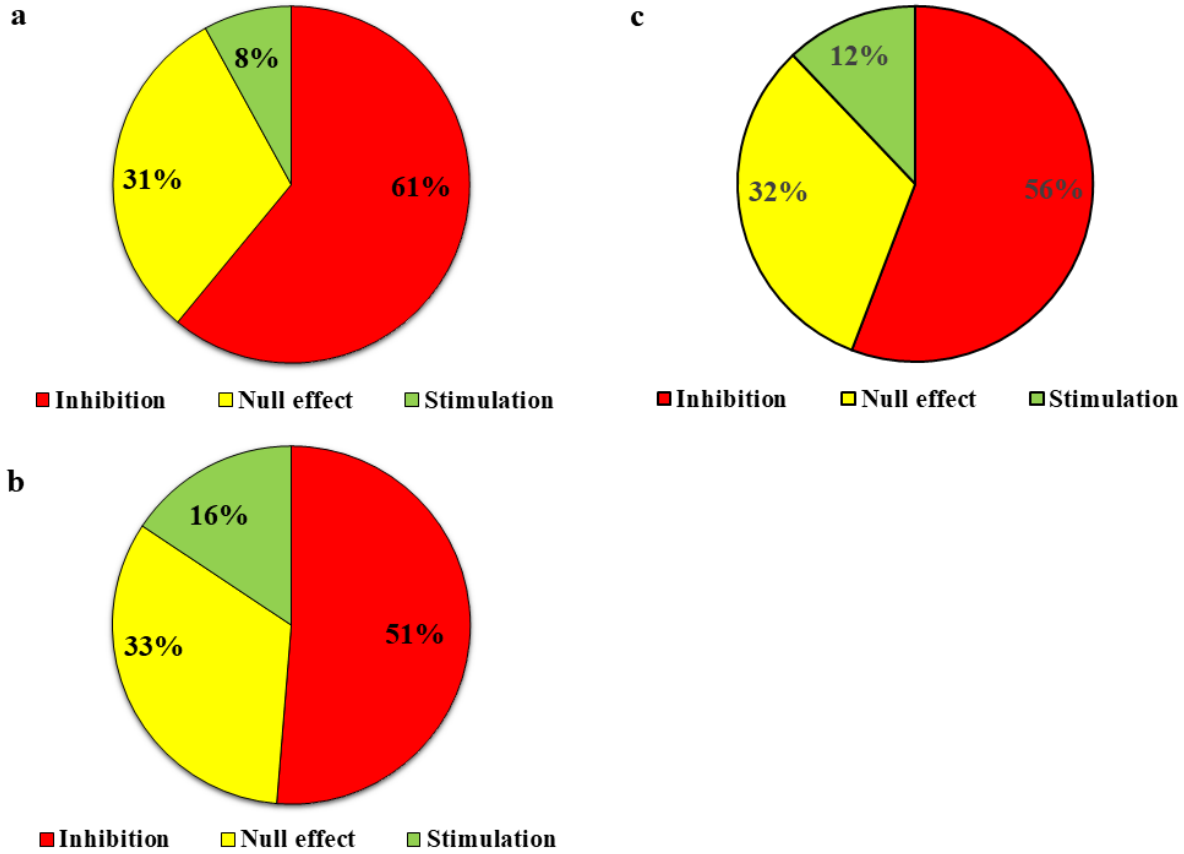


Fig. 14. Percentage of effects (inhibition, null effect and stimulation) by **(a)** systemic, **(b)** contact and **(c)** whatever mode of action of fungicides on arbuscular mycorrhizal (AM) fungi under greenhouse or field conditions. Data based on 117 scientific articles published between 1977-2021.

Herbicides are the most widely used class of pesticides in agriculture. They are phytotoxic chemicals used for destroying weeds or inhibiting their growth with variable degrees of specificity (Gupta 2017). The impacts of herbicides (e.g., glyphosate) on AM fungi have been shown in many studies (Trappe et al. 1984; Ellouze et al. 2008; Hage-Ahmed et al. 2019) (**Table 4**).

Table 4. Impacts of glyphosate on arbuscular mycorrhizal fungi.

Herbicide	Concentration	AM Fungi	Host plant	Culture conditions	Principal effects on AM fungi	Reference
Cellular metabolism						
Inhibition of Enolpyruvyl Shikimate Phosphate Synthase						
Glyphosate	0.4, 2.0, 4.0 L ha^{-1}	*F. mosseae*	*Glycine max*	Pot	Null effect on RC	Mujica et al. 1999
	0.62 kg ha^{-1}	Indigenous AM fungi	*Vitis vinifera*	Field	Null to inhibitory effect on RC according to seasonal changes and treatments (post-emergence or pre-emergence)	Baumgartner et al. 2005
	1.25, 2.5, 5.0, 7.5, 10 L ha^{-1}; 25,6, 51,0, 102,0, 204,5, 409 µmol L^{-1}	*G. margarita* *C. etunicatum* *S. heterogama*	*Glycine max*	Greenhouse/*in vitro*	Null effect on RC (greenhouse). Inhibition of growth of AM fungi under increasing doses (*in vitro*)	Malty et al. 2006
	0.63 µM, 3.15 µM, 6.32 µM	*F. mosseae*	*Capsicum annuum*	Growth chamber	Inhibition of RC under increasing doses. Null effect on ALP in roots	Ronco et al. 2008
	0.9, 1.8 kg ha^{-1}	*R. intraradices*	*Glycine max*	Greenhouse	Null effect on RC	Powell et al. 2009
	1.1, 2.2, 3.3, 21.6 µg g^{-1}	*F. mosseae*	*Arachis hypogaea*	Glasshouse	Inhibition of RC under increasing doses	Pasaribu et al. 2011
	3.6 g L^{-1}	Indigenous AM fungi	*Triticum aestivum*	Greenhouse	Null effect on RC	Brito et al. 2013
	0.8, 3.0 L ha^{-1}		*Paspalum dilatatum* *Lotus tenuis*		Inhibition of RC and spore viability under increasing doses	Druille et al. 2013a
	0.8, 3.0 L ha^{-1}		*Lolium multiflorum*		Inhibition of RC and spore viability under increasing doses	Druille et al. 2013b
	720 g ha^{-1}		*Glycine max*	Field	Inhibition or null effect on RC according to commercial formulation	Reis et al. 2014
	7.2 g L^{-1}	*F. mosseae*	*Trifolium repens*	Greenhouse	Inhibition of RC and SP	Zaller et al. 2014
	1.44, 3.84 g ha^{-1}	Indigenous AM fungi	Native grasslands	Field	Inhibition of spore viability	Druille et al. 2015
	1.68 kg ha^{-1}		*Brassica nigra*		Increase of HL	Maltz et al. 2016
	2.25 kg ha^{-1}		*Festuca pratensis* *Elymus repens*		Inhibition of RC	Helander et al. 2018
	10, 50, 75, 100, 150, 200, 250, 300, 350 g L^{-1}		*Triticum aestivum*	Glasshouse	Decreased ergosterol and arbuscules with conventional tillage	Wilkes et al. 2021
	6.0, 12, 18, 24 ml L^{-1}	AM inoculum	*Abelmoschus esculentus*	Field	AM fungi promoted dissipation of glyphosate at low concentration.	Brindhavani et al. 2020
	10, 50, 100, 250, 500, 750 and 1000 mg kg^{-1}	*G. albida* *R. clarus*	NM	Growth chamber	Null effect on SG of *G. albida* Inhibition of SG of *R. clarus*	Malfatti et al. 2021

Abbreviations: RC: Root colonization, **SP:** Spore production, **SG:** Spore germination, **EA:** Enzymatic activity, **ALP**: alkaline phosphatase, **HL:** hyphal length, **NM:** Not mentioned.

Herbicides could impact AM fungi either directly by contact (Pasaribu et al. 2011; Li et al. 2013) or indirectly, by exerting phytotoxicity to their host plant (Druille et al. 2013a). However scientific knowledge on this interaction remains limited (Malfatti et al. 2021).

II.4.2.1. Systemic fungicides

Systemic curative fungicides move into the plant vascular system either by penetrating through the leaf surface and/or by being absorbed by the roots (Morton and Staub 2008, Mabbett 2018). Several studies have suggested that systemic fungicides impact more AM fungi than contact fungicides. It has been hypothesized that this was because compounds of systemic fungicides accumulate in or on the roots in contrast to contact fungicides (Burrows and Ahmed 2007; Diedhiou et al. 2004; Jin et al. 2013; Buysens et al. 2015). However, redistribution in the plant vascular system is a complex attribute of these fungicides. The redistribution is influenced by formulation, plant characteristics, and environmental conditions as well as by the physical properties of each active ingredient (Klittich and Ray 2013; Klittich 2014).

Soil fumigation has often been used to suppress the activity of indigenous AM fungi in several experiments for the establishment of a non-mycorrhizal control treatment (O'Connor et al. 2009). Under field conditions, fungicides do not completely eradicate mycorrhizal colonization of plants but decrease development for a variable time after application (Allsup and Piege 2016). For example benomyl, a broad-spectrum and systemic benzimidazole that disrupts ß-tubulin assembly in mitosis (Fungicide Resistance Action Committee 2022), has been the most widely used fungicide for AM fungi suppression in scientific research because it consistently inhibits AM colonization (Blanke et al. 2011; Zhang et al. 2011; Yu et al. 2012; Knappová et al. 2016; Yang et al. 2018), ERM development (Sukarno et al. 1993; Kjøller and Rosendahl 2000; Wilson et al. 2009) and spores production in the field (Mei et al. 2019).

Under greenhouse conditions, a strong reduction in AM fungal colonization by soil drenching has also been observed (Yu et al. 2012; Wang et al. 2018; Pereyra et al. 2019). Benomyl was even shown to suppress other types of non-target rhizospheric fungi (e.g., dark septate endophytes) (Grilli et al. 2014). This fungicide often showed little or no direct effects on the non-mycorrhizal (NM) plant and bacterial community (Kähkölä et al. 2012; Zhang et al. 2016) and was rapidly degraded in the soil (O'Connor et al. 2009). In general, soil drench with benomyl is preferable to a foliar spray to reduce AM fungal development (Pedersen and Sylvia 1997). Benomyl is currently used in several studies around the world focused on AM fungi. However, the company Dupont ceased its production (benlate®) in 2001. This compound is thus no longer available commercially (Wilson and Williamson 2008).

Thiophanate methyl is a broad-spectrum systemic fungicide, recommended as substitute to benomyl, due to the similar mode of action and the effectiveness to reduce AM fungal colonization with no phytotoxic effects (Wilson and Williamson 2008; Rajeshkannan et al. 2009; McCain et al. 2011; Paudel et al. 2014). A similar effect was observed in combination with carbendazim applied on the soil (Karanika et al. 2008; Rajeshkanna et al. 2009).

In general and whatever the method of application, systemic fungicides impact the development of AM fungi (Boatman et al. 1978, Nemec 1980; Ocampo and Hayman 1980; Jabaji-Hare and Kendrick 1987; Sukarno et al. 1993; Vijayalakshmi and Rao 1993; Sugavanam et al. 1994; Seymour et al. 1994; Carrenho et al. 1998; Fontanet et al. 1998; Sukarno et al. 1998; Venedikian et al. 1999; Carrenho et al. 2000; Kjøller and Rosendahl 2000; Bødker et al. 2002; Aggarwal et al. 2005; Rao et al. 2006; Murillo-Williams and Pedersen 2008; Assaf et al. 2009; Hernández-

Dorrego and Mestre-Parés 2010; Jin et al. 2013; Channabasava et al. 2015; Mallmann et al. 2018; Pánková et al. 2018; de Novais et al. 2019a, 2019b). Although, a high variation on fungicide impact was observed in function of AM fungal species or plant host species (Dodd and Jeffries 1989; Plenchette and Perrin 1992; von Alten et al. 1993; Seymour et al. 1994; Burrows and Ahmed 2007; Fontanet et al. 1998; Udaiyan et al. 2001). These results indicate that suppressive effects of fungicides on AM fungi development depend on specific interactions between fungicides and mycorrhizal partners (Jin et al. 2013). Furthermore, systemic fungicides often showed less or transitory impacts on symbiosis when plants were naturally colonized in field or inoculated with various AM fungi under controlled conditions (von Alten et al. 1993; Carrenho et al. 1998, 2000; Marin et al. 2002; Bary et al. 2005; Ipsilantis et al. 2012). Studies suggested a differential tolerance of AM fungi to the fungicides.

Interestingly, stimulatory effects on AM fungi were noticed by application of systemic fungicides used in crops to control oomycetes (e.g., metalaxil, propamocarb and fosetyl-al) (Nemec 1980; Groth and Martinson 1983; Jabaji-Hare and Kendrick 1987; Afek et al. 1990, 1991; Hetrick and Wilson 1991; Plenchette and Perrin 1992; Shetty and Magu 1997; Fontanet et al. 1998; Marin et al. 2002; Rabad and Reda 2009; Hernández-Dorrego and Mestre-Parés 2010). Some authors hypothesized that the increase in root colonization by AM fungi after soil application with fungicides could be related to decrease in competition exerted by soil fungal pathogens (Jabaji-Hare and Kendrick 1987; Afek et al. 1990, 1991; von Alten et al. 1993; Murillo-Williams and Pedersen 2008). In addition, carbendazim, a systemic benzimidazole with broad-spectrum, increased root colonization at low doses but inhibited the symbiosis at high concentrations (Saleh and Sikora 1988; Vijayalakshmi and Rao 1993; Schweiger et al. 2001; Kumar et al. 2005; Ipsilantis et al. 2012). Recently, this biphasic response to carbendazim was confirmed in three species of AM fungi, suggesting a hormesis effect on root colonization (Jakobsen et al. 2021). The hormesis is a biphasic dose response characterized by temporal stimulation at low doses and inhibition at high doses of a stressor agent, suggesting an adaptive response that can be either directly induced or be the result of overcompensation after the alteration of homeostasis in a biological process (Calabrese and Baldwin 2002). The hormetic effects of fungicides on mycelial growth and virulence have been previously reported in Oomycota, Ascomycota and Basidiomycota plant pathogens (Garzon and Flores 2013; Lu et al. 2018; Zhang R. et al. 2019), but in Glomeromycota, the knowledge is scarce and the mechanisms involved remains to be elucidated.

Fungicide resistance refers to an acquired, heritable reduction in sensitivity of a fungus to a specific fungicide (Fungicide Resistance Action Committee 2022). Fungal pathogens from Ascomycota, Basidiomycota and Deuteromycota and fungal-like species from the Oomycota can become resistant to fungicides through different mechanisms. These mechanisms include: (i) an altered target site, which reduces the binding of the fungicide; (ii) the synthesis of an alternative enzyme capable of substituting the target enzyme; (iii) the overproduction of the fungicide target; (iv) an active efflux or reduced uptake of the fungicide; and (v) a metabolic breakdown of the fungicide (Ma and Michailides 2005).

To date, scientific reports of fungicide resistance mechanisms in Glomeromycota members have not been addressed. However, several reports elucidate potential mechanisms of resistance to fungicides. Furthermore, other authors hypothesized that AM fungi enzymatic pathways, could be less sensitive to fungicides than those of phytopathogenic fungi (Buysens et al. 2015).

II.4.2.2. Contact fungicides

Contact fungicides protect plant tissues superficially and are not taken up into the plant tissue. Captan, a broad spectrum phthalimide, is the most important fungicide of this class. It has been considered as an alternative to create a non-mycorrhizal treatment due to a marked decrease of AM fungal colonization or spore production in experiments conducted in soil (Nemec 1980; Manjunath and Bagyaraj 1984; Kough et al. 1987; Spokes et al. 1989; Screenivasa and Bagyaraj 1989; Salem and Dobolyi 2003; Al-Garni 2006; Burrows and Ahmed 2007; Kangwankraiphaisan et al. 2013). This fungicide persists in the environment for only two weeks (Allsup and Paige 2016).

Several studies have reported a negative impact on AM fungi by application of multi-site mode of action fungicides in soil (e.g., dithiocarbamates, chloronitriles and sulfamides) (Spokes et al. 1989; Sreenivasa and Bagyaraj 1989; Aziz et al. 1991; Plenchette and Perrin 1992; Sugavanam et al. 1994; Aggarwal et al. 2005; Kumar et al. 2005; Zhang et al. 2006; Hernández-Dorrego and Mestre-Parés 2010; Campos et al. 2015; Channabasava et al. 2015; Mallmann et al. 2018). Likewise, soil application of quintozene (PCNB) and etridiazole that affect cell peroxidation, was shown to decrease AM fungal development (Menge et al. 1979; Saleh and Sikora 1988; Sreenivasa and Bagyaraj 1989; Gnekow and Marschner 1989; Schüepp and Bodmer 1991; Pattinson et al. 1997). Mancozeb a dithiocarbamate, inactivates sulfhydryl groups and hence disrupts lipid metabolism, respiration and adenosine triphosphate production (Jakobsen et al. 2021). It negatively affects AMF spore germination. (Mallmann et al. 2018) and root colonization (Hernandez-Dorrego and Pares, 2010; Channabasava et al. 2015; Vuyyuru et al. 2018).

Mostly, a null or transitory effect was noticed, following seed or foliar application of contact fungicides (Spokes et al. 1989; Gonçalves et al. 1991; Muchovej et al. 1991; Plenchette and Perrin 1992; Burrows and Ahmed 2007; Williams and Pedersen 2008; Jin et al. 2013; Castelli et al. 2014; Grageda-Cabrera et al. 2014), or prior to seed sowing and during seedling emergence (Bertoldi et al. 1977; Vijayalakshmi and Rao 1993). Other studies demonstrated that the differential impact (negative to null effect) of contact fungicides was determined by host plant species or AM fungi inoculated whatever the mode of application (Plenchette and Perrin 1992; Schreiner and Bethlenvay 1997; Udaiyan et al. 2001; Burrows and Ahmed 2007). Interestingly, stimulation to neutral effects on AM fungal life cycle was observed under increasing doses of contact fungicides (Menge et al. 1979; Nemec, 1980; Vijayalakshmi and Rao 1993; Marin et al. 2002). Lately, a hormetic effect was suggested on root colonization of *Glomeraceae* species following application of mancozeb (Jakobsen et al. 2021).

The potential negative effects of systemic and contact fungicides on non-target AM fungi are not fully understood and are influenced by several factors (Jin et al. 2013). For instance, the period of application (early or late stage of plant development), the mode of application (leaf application, seed treatment or soil drench) and could modify the impact of fungicides on AM fungi irrespective the mode of absorption (systemic or contact).

II.4.3. Screening effects of pesticides under *in vitro* conditions

In vitro cultivation systems offer several advantages to study the physiology of ERM in presence of stressor agents, among which, the absence of any confounding effects caused by unwanted contaminants or environmental factors (e.g., soil physico-chemical parameters) and the easy non-destructive observations of growth and development of fungi (Buysens et al.

2015). Different fungal parameters as spore germination (Chiocchio et al. 2000; Zocco et al. 2008; Buysens et al. 2015), root colonization (Campagnac et al. 2008, 2009, 2010; Calonne et al. 2010, 2012), anastomosis formation (Cardenas-Flores et al. 2011), enzymatic activity (Campagnac et al. 2010; Calonne et al. 2012) and transport of nutrients (e.g., phosphorus) from fungus to plant (Zocco et al. 2011) have been investigated *in vitro* in presence of different types of fungicides (**Table 5**).

Table 5. Impact of fungicides on arbuscular mycorrhizal (AM) fungi investigated under *in vitro* culture conditions using root organs cultures (ROC) or whole plants.

Fungicide	Concentration mg L^{-1}	AM fungi	Host plant	Principal effects in symbionts	Reference
B: Cytoskeleton and motor protein					
Benomyl Benzimidazoles	0.001, 0.003, 0.006, 0.013, 0.025 mg L^{-1}	*R. irregularis* MUCL 43194/ DAOM 197198 = DAOM 181602	*Daucus carota* (Ri T-DNA transformed roots)	Inhibition of RC under increasing concentrations	Wan et al. 1998
	0.001, 0.01, 0.1, 2.12, 10.62 µg ml^{-1}	*F. mosseae*	NM	Inhibition of SG and HL under increasing concentrations	Chiocchio et al. 2000
Carbendazim Benzimidazoles	12.5, 25 nl ml^{-1}	*F. mosseae* *F. geosporus* *F. monosporum*		Inhibition of SG	Dodd and Jeffries 1989
	0.01, 0.1, 1.0, 25, 120, 250 µg ml^{-1} x 10^3	*F. mosseae*		Inhibition of SG and HL under increasing concentrations	Venedikian et al. 1999
	0.1, 1.0, 10 mg L^{-1}	*C. etunicatum*	*Daucus carota* (Ri T-DNA transformed roots)	Inhibition of SG and HL under increasing concentrations	Gong et al. 2014
Pencycuron Phenylureas	0.1, 0.5, 1.0, 5.0, 10, 100 mg L^{-1}	*R. irregularis* MUCL 41833/	*Solanum tuberosum* (Whole plant)	Inhibition of SG and RC under increasing concentrations Null effect on SP and ERM	Buysens et al. 2015
C: Respiration					
Azoxystrobin Methoxyacrylates	0.1, 1.0, 10, 100 mg L^{-1}	*R. irregularis* MUCL 41833/ Whole plant	*Solanum tuberosum* (Whole plant)	Inhibition of SG, RC, SP and ERM under increasing concentrations	Buysens et al. 2015
Flutolanil Phenylbenzamides				Inhibition of SG and RC under increasing concentrations Null effect on SP and ERM	
G: Sterol Biosynthesis in membranes					
Triadimefon Morpholines	25, 50 nl ml^{-1}	*F. mosseae* *F. geosporus* *F. monosporum*	NM	Null effect on SG (*F. mosseae*, *F. monosporum*) Decreased SG at high concentration (*F. geosporus*)	Dodd and Jeffries 1989
Fenpropimorph Morpholines	0.02, 0.2, 2.0 mg L^{-1}	*R. irregularis* MUCL 43194/ DAOM 197198 = DAOM 181602	*Daucus carota* (Ri T-DNA transformed roots)	Decreased root growth, root colonization, ERM and modified sterol content under increasing concentrations	Campagnac et al. 2008

Fenpropimorph Morpholines	0.02, 0.2, 2.0, 20 mg L^{-1}		*Chicorium intybus* (Ri T-DNA transformed roots)	Decreased sterols content and stimulateion of the amount of squalene Inhibition of SP, HL and fungal biomass under increasing concentrations	Campagnac et al. 2009
	0.2, 2.0 mg L^{-1}			Inhibition of HL and sterol content with both concentrations. Stimulated squalene content at 2 mg L^{-1}. The gene C-4 sterol methyl oxidase was activated for the long-time exposure	Oger et al. 2009
	0.02, 0.2 mg L^{-1}			Inhibition of RC, HL and SP Caused oxidative damage by increasing lipid peroxidation	Campagnac et al. 2010
	10 mg L^{-1}		*Daucus carota* (Ri T-DNA transformed roots)	Caused an oxidative stress in ERM 7 and 14 days after exposure and induced a considerable increase in lipid peroxidation	González-Guerrero et al. 2010
	0.2, 2.0 mg L^{-1}	*Glomus* sp. MUCL 43204	*Medicago truncatula* (Whole plant)	Decreased P transport and EA activities on ERM.	Zocco et al. 2011
Fenhexamid Hydroxyanilides	0.2, 2.0, 20, 200 mg L^{-1}	*R. irregularis* MUCL 43194/ DAOM 197198 = DAOM 181602	*Daucus carota* (Ri T-DNA transformed roots)	Inhibition of SG and HL by increasing concentrations. ERM and SP were completely inhibited at 20 and 200 mg L^{-1}.	Zocco et al. 2008
	0.02, 0.2, 2.0 mg L^{-1}			Modification of sterol profiles, inhibition of RC.	Campagnac et al. 2008
	0.02, 0.2 mg L^{-1}		*Chicorium intybus* (Ri T-DNA transformed roots)	Oxidative damage by increasing lipid peroxidation and inhibition of HL and SP	Campagnac et al. 2010
	0.5, 10 mg L^{-1}	*R. clarum* MUCL 46238	*Daucus carota* (Ri T-DNA transformed roots)	Inhibition of hyphal anastomosis and impact on the morphology of the germ tubes at 10 mg L^{-1} Null effect on SP	Cardenas-Flores et al. 2011
	0.2, 2.0 mg L^{-1}	*Glomus* sp. MUCL 43204/Whole plant	*Medicago truncatula* (Whole plant)	Inhibition of EA activities of ERM Inhibition of RC, SP and ERM under increasing concentrations	Zocco et al. 2011
Propiconazole Triazoles	0.02, 0.2, 2.0 mg L^{-1}	*R. irregularis* MUCL 43194/ DAOM 197198 = DAOM 181602	*Chicorium intybus* (Ri T-DNA transformed roots)	Inhibition of SG, HL, RM, ERM and SP under increasing concentrations	Calonne et al. 2010
				Inhibition of the sterol 14α-demethylase, SP and HL	Calonne et al. 2012

M: Chemicals with multi-site activity

Copper sulfate Inorganic	0.13, 0.63, 1.25, 6.25, 12.5 mg L^{-1}	*R. irregularis* MUCL 43194/ DAOM 197198 = DAOM 181602	*Daucus carota* (Ri T-DNA transformed roots)	Inhibition of RC under increasing concentrations	Wan et al. 1998
Chlorothalonil Chloronitriles	0.01, 0.06, 0.13, 0.63, 1.25 mg L^{-1}			Inhibition of RC at 0.13, 0.63, 1.25 mg L^{-1}	
	0.000001, 0.0001, 0.01, 1.0, 12, 64, 120 µg ml^{-1} x 10^3	*F. mosseae*	NM	Inhibition of SG and HL under increasing concentrations	Venedikian et al. 1999
Thiram Dithiocarbamates	0.1, 1.0, 10 mg L^{-1}	*C. etunicatum*	*Daucus carota* (Ri T-DNA transformed roots)	Inhibition of SG and HL under increasing concentrations	Gong et al. 2014

Abbreviations: RC: Root colonization, **SP:** Spore production, **SG:** Spore germination, **EA:** Enzymatic activity, **HL:** hyphal length, **NM:** Not Mentioned.

The results under *in vitro* culture conditions differed significantly between fungicides, AM fungi and doses of application. For example, carbendazim decreased spore germination of *F. mosseae* and *F. geosporum* but not spore germination of *F. monosporum* (Dodd and Jeffries 1989). In the same study tridemorph did not affect any AM fungi. In addition, increasing concentrations of benomyl, chlorothalonil and copper sulfate, decreased root colonization by *R. intraradices* (Wan et al. 1998) and benomyl inhibited spore germination and hyphal length of *F. mosseae* (Chiocchio et al. 2000) and *F. caledonius* (Carr and Hinkley 1985). A similar trend was observed with chlorothalonil, carbendazim and thiram, impacting spore germination, hyphal length and enzymatic activity as well as succinate dehydrogenase and alkaline phosphatase activity of *C. etunicatum* (Gong et al. 2004).

Mainly, sterol biosynthesis inhibitors fungicides (e.g., fenpropimorph, fenhexamid) have been evaluated under ROC conditions with *Glomeraceae* strains. The effects of fenpropimorph and fenhexamid on the AM fungal symbiosis was demonstrated using ROC of *Daucus carota* and *Cichorium intybus* (Campagnac et al. 2008, 2009, 2010; Zocco et al. 2008; Oger et al. 2009) or whole plants (e.g., *Medicago truncatula*) (Zocco et al. 2011). These sterol biosynthesis inhibitors fungicides presented a high toxicity with drastic sterols modifications in the host roots which was mirrored by a drastic reduction of root growth, root colonization (Campagnac et al. 2008). For instance, fenpropimorph even at lowest concentration inhibited spore production, hyphal growth, and fungal biomass of *R. irregularis* (Campagnac et al. 2009). Same authors noticed that the sterol pathway was altered by a decrease in total sterols, an increase in the amount of squalene and noaccumulation of abnormal sterols. Furthermore, fenpropimorph and fenhexamid cause oxidative damage by increasing lipid peroxidation measured by malondialdehyde production in chicory roots in absence of *R. intraradices* (Campagnac et al. 2010). Last authors mentioned that the presence of the AM fungus can alleviate the fungicide impact. Same fungicides mostly fenpropimorph decrease phosphorus transport, alkaline phosphatase and succinate dehydrogenase activities of the ERM of *Glomus* sp. MUCL 43204 associated with *M. truncatula* (Zocco et al. 2011). Interestingly, fenpropimorph also produced an induction of oxidative stress at high concentration (10 mg L^{-1}), suggesting the perturbation of other biological processes such as response to biotic and abiotic environmental stimuli (González-Guerrero et al. 2010). In addition, a high dose of fenhexamid (10 mg L^{-1}) impacted the anastomosis process in *R. clarum*, reducing the hyphal fusion between germinating spores (Cardenas-Flores et al. 2011). Moreover, concentrations of propiconazole impacted AM fungal life cycle of *R. irregularis* (Calonne et al. 2010) and spore germination of *Funneliformis* species (Dodd and Jeffries 1989). Propiconazole decreased the development cycle (germination, germ tube elongation, colonization, extraradical hyphal growth) of *R. irregularis*. The authors related this effect to (i) an inhibition of sterol 14a-demethylase activity, a key enzyme located in the sterol biosynthesis pathway of *R. irregularis* associated with chicory roots like in phytopathogenic fungi. This resulted in accumulation of sterols precursors and decrease of the production of end-chain sterols (Calonne et al. 2012), (ii) the disturbance of lipid metabolism (fatty acids, phospholipids and their associated fatty acids) and to the toxicity of malondialdehyde accumulation caused by the peroxidation of membrane lipids (Calonne et al. 2010).

More recently, the impact of the systemic fungicides azoxystrobin, flutolanil, and the contact fungicide pencycuron on AM fungi were analyzed using whole plants (*Solanum tuberosum*). Azoxystrobin and its formulation Amistar ® did not impact spore germination and root colonization of *R. irregularis* at the threshold concentration (IC50 $\leq$ 0.1 mg L^{-1} a.i.) used for the control of *Rh. solani*, while at 10 times this threshold, spore production and mycelium development were strongly affected (Buysens et al. 2015). In the same study, at threshold value

for the control of *Rh. solani in vitro*, pencycuron (0.010 mg L^{-1} a.i.) and its formulation Monceren (0.009 mg L^{-1} a.i.), did not affect spores germination and intra- or ERM development (Buysens et al. 2015). The same authors noticed that flutolanil (0.13 mg L^{-1} a.i.) and its formulation Monarch (0.15 mg L^{-1} a.i.) at threshold value for the control of *Rh. solani in vitro* did not affect spores germination or ERM development but decreased root colonization and arbuscules formation.

Additionally, differential effects of herbicides on the AM fungal growth were observed under *in vitro* conditions. Negative, neutral, or positive effects under increasing doses of herbicides were noticed on spores germination of *Glomeraceae* members (Dodd and Jeffries 1989). Dichlobenil and paraquat decreased hyphal length of *R. intraradices* and *Glomus versiforme* by increasing doses (Hamel et al. 1994). In the same study simazine did not affect hyphal length. More recently, glyphosate above recommended field doses (53.6 and 322.9 μM, respectively) reduced strongly germination of spores and growth of germ tubes of AM fungi (e.g., *C. etunicatum* and *S. heterogama*) (Malty et al. 2006). A similar trend was observed with prometryn on spore germination of *C. etunicatum* at a concentration of 10 mg L^{-1} a.i., while acetochlor did not impact spores germination. Both herbicides decreased root colonization, spore production and succinate dehydrogenase activity (Li et al. 2013).

The impact of herbicides and fungicides on AM fungi are mainly based on measuring root colonization and spore production or germination of *Glomeraceae* members. In general, studies of the effects of these pesticides on important growth and adaptation mechanisms such as anastomoses are scarce. Indeed, recent studies under growth chamber conditions demonstrated a strong impact on anastomosis and ERM viability of *F. mosseae* by application of fungicides (e.g., fenhexamid and benomyl) and herbicides (e.g., dicamba, glufosinate-ammonium and pendimethalin) (de Novais et al. 2019a, 2019b). However, important AM fungal groups such as *Gigasporaceae* have not been studied under pesticide exposure.

II.4.4. Impact of pesticides on the Pi uptake by mycorrhizal plants

Pesticides could affect AM fungal activity directly through the soil or indirectly through systemic responses in the plant (Kjøller and Rosendahl 2000). The impacts of fungicides on P transport by AM fungi have been reported in many studies. For example, fungicide suppression of AM fungal Pi uptake was observed with application of benomyl (Boatman et al. 1978; Sukarno et al. 1996; Larsen et al. 1996; Kjøller and Rosendahl 2000), thiophanate-methyl (Boatman et al. 1978) and carbendazim (Kling and Jakobsen 1997). Conversely, Pi uptake by AM fungi appeared to be stimulated at very low concentrations of carbendazim (Schweiger and Jakobsen 1998) and under the recommended field application of commercial formulation of this compound (Schweiger et al. 2001). Lately, carbendazim and mancozeb generated a hormetic response (stimulating at low and inhibiting at high concentration) on the hyphal length-specific ^{33}P uptake of *F. caledonium* and *C. claroideum* in symbiosis with pea (Jakobsen et al. 2021). The same authors reported a similar trend on shoot P content in presence of *C. claroideum*.

Moreover, sterol biosynthesis inhibitors fungicides as fenpropimorph inhibited Alkaline Phosphatase activity in internal and external hyphae of *F. caledonius* at high application level (125 mg g^{-1}) (Kjøller and Rosendahl 2000). In contrast, the same study demonstrated that propiconazole decreased the activity of the external hyphae at low application level (0.21 mg g^{-1}) but did not affect the internal activity at any application level. The last authors suggested that external hyphae could be more sensitive to the direct contact with fungicides than internal

hyphae as the root surface may protect the latter. Furthermore, using a whole plant under *in vitro* conditions, fenpropimorph and fenhexamid have been reported to impact the transport of P from fungus to plant (Zocco et al. 2011).

The impact of herbicides on Pi uptake by the AM fungi has been scarcely studied. For example, increasing doses of glyphosate did not affect alkaline phosphatase in pepper roots colonized by *F*. *mosseae* (Ronco et al. 2008). Conversely, under *in vitro* culture conditions, increasing doses of prometryn and acetochlor decreased alkaline phosphatase in Ri T-DNA hairy roots of carrot associated with *C. etunicatum* (Li et al. 2013). The nutritional benefits provided by AM fungi to host plants have been demonstrated frequently on several crop species such as potato. However, symbiosis with these microorganisms may be threatened by the continuous application of pesticides, which remains the principal strategy against pests in crops (Hage-Ahmed et al. 2019).

III. RESEARCH OBJECTIVES

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with many plant species, including most crops. They provide multiple benefits to their hosts (e.g., increased nutrients uptake, drought resistance and protection against pests and diseases) (Chen et al. 2018) and are thus considered essential microorganisms that help feed the world. In this symbiosis, the AM fungi transfer mainly inorganic phosphorus (Pi) to their host plants in exchange for carbon (C) resources produced during photosynthesis (Fellbaum et al. 2012). The arbuscules, within the root cells, are the principal site of C-P exchange, while the extraradical mycelium (ERM) network developing from roots into the soil, transport nutrients to the arbuscules (Pepe et al. 2018). The ERM is the sum of innumerable hyphae interconnecting each other via a mechanism named anastomosis, to form a wide network reaching up to 100 m per g of soil (Mikkelsen et al. 2008). It involves different branches of the same or different hyphae which fuse to constitute the mycelial network (Voets et al. 2006). The anastomosis also concerns the recovery of hyphae integrity after physical damage. This process has been described as a hyphal healing mechanism (HHM) and was shown to differ between the *Glomeraceae* and *Gigasporaceae* (de la Providencia et al. 2005, 2007), two major phyla of the Glomeromycota.

Numerous studies have suggested that AM fungi in agroecosystems are affected by a wide range of agricultural practices (e.g., tillage, monoculture, fertilization) (Rivera-Becerril et al. 2017; Soka and Ritchie 2018; Chagnon et al. 2022). Among these practices, the widely used plant protection products (e.g., fungicides and herbicides), to control pests, diseases, and weeds, deserve particular attention owing to their often broad-scale activity impacting also non-target microorganism such as AM fungi. Several studies have demonstrated that these chemicals impact directly or indirectly the AM symbiosis (Hage-Ahmed et al. 2018) However, the knowledge on the impact of these chemicals on the anastomosis (including the HHM) mechanism is extremely limited. In addition, the effects of fungicides on the dynamics of Pi uptake by mycorrhizal plants are poorly understood.

The first objective of the PhD was to investigate under *in vitro* culture conditions the impact of azoxystrobin, pencycuron, flutolanil, and fenpropimorph at two different concentrations (0.02 and 2 mg L^{-1}) on the HHM in *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833. This objective was completed by an experiment led in in vitro conditions (ROC), in which the AM fungi was associated to a transformed root of chicory (**Chapter 1**).

Glyphosate, the world's most widely used pesticide, specifically inhibits the 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme of the shikimate-pathway in plant photosynthesis (Duke and Powles 2008). Although, differential effects on non-target organisms (e.g., bacteria, annelids, and fungi) have been noticed (Schlatter et al. 2017; Zaller et al. 2021). Indeed, contrasting effects on AM fungi were noticed under greenhouse or field conditions (Brito et al. 2013, Druille et al. 2013a,b; Druille et al. 2015; Helander et al. 2018; Wilkes et al. 2021). In general, the direct effect of the glyphosate on AM fungi has not been fully understood. **The second objective** of the PhD was thus to study under strict *in vitro* culture conditions the impact of the glyphosate on the HHM in *Gigasporaceae* and *Glomeraceae* members. For this, an experiment led in in vitro conditions (ROC), in which the AM fungi was associated to a transformed root of chicory was used (**Chapter 2**).

Interestingly, *Glomeraceae* members can interconnect within and between hyphal networks belonging to the same isolate (Voets et al. 2006), while this was seldom observed (at least for different networks) for *Gigasporaceae*. Recently, it was demonstrated that fungicides and

herbicides are detrimental to anastomosis in *Funneliformis mosseae* under growth chamber conditions (de Novais et al. 2019a, 2019b).

The third objective of the PhD was thus to investigate under *in vitro* culture conditions the impact of the same four pesticides as in the first objective at two concentrations (0.02 and 2 mg L^{-1} a.i.) on the capacity of *R. irregularis* to form anastomoses within and between ERM networks of the same isolate of *R. irregularis* MUCL 41833. To complete this objective, a quadri-compartmental ROC system containing two root compartments and a central compartment (hyphal compartment) allowed the observation of anastomonis formation in this hyphal compartment (**Chapter 3**).

Azoxystrobin is globally used to control several fungal pathogens in crops (Bartlett et al. 2002; Feng et al. 2020). However, this fungicide impacts non-target beneficial soil organisms such as AM fungi. Previous studies have shown a negative effect on AM symbiosis under *in vitro* (Buysens et al. 2015) and greenhouse conditions (Diedhiou et al. 2004; Vuyyuru et al. 2018). However, its impact on the plant nutrients uptake by AM symbiosis at the recommended dosage in field has not been studied.

The fourth objective of the PhD was thus to evaluate under greenhouse conditions the dynamics of Pi uptake of potato plants associated or not to *R. irregularis* MUCL 41833 and grown in presence or absence of azoxystrobin. This objective was completed performing a circulatory semi-hydroponic cultivation system under greenhouse conditions (**Chapter 4**).

To answer the objectives, four specific questions were addressed:

1. What is the impact of different classes of fungicides on the HHM in *Gigasporaceae* and *Glomeraceae*?

2. What is the impact of glyphosate on the HHM in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331, two AM fungi with different life history strategies?

3. How does different classes of fungicides affect the anastomosis process within and between networks of *R. irregularis* MUCL 41833?

4. Is the dynamics of Pi uptake by potato plants grown in presence of azoxystrobin more efficient when associated to *R. irregularis* MUCL 41833 than in absence of the fungus?

IV. MATERIALS AND METHODS

IV.1. Biological material

IV.1.1. Arbuscular mycorrhizal fungi

Rhizophagus irregularis (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] MUCL 41833, formerly named *Glomus intraradices*/*irregulare* (**Figure 15**) (Redecker et al. 2013; Walker et al. 2021) was isolated from a banana field in the Canary Islands, Spain (Declerck et al. 1998). This AM fungal strain is a model organism used in several studies under greenhouse (Garcés-Ruiz et al. 2017; Calonne-Salmon et al. 2018; Le Pioufle et al. 2019; Feldmane et al. 2021) and *in vitro* (Buysens et al. 2015; Le Pioufle and Declerck et al. 2019; El Hilali et al. 2021) conditions. Remarkably, this AM fungal species is associated with numerous plant species and distributed all over the world. Currently, *R. irregularis* is the model species for AM fungi research and the most widely propagated species for commercial plant biostimulants (Kokkoris et al. 2023). In recent years through phylogenetic analysis, it has been demonstrated that *R. irregularis* is the most predominant AM taxon in crop soils regardless of commercial inoculation (Savary et al. 2018; Renaut et al. 2020; Austen et al. 2022). It is considered a generalist symbiont and r-strategist (IJdo et al. 2010).

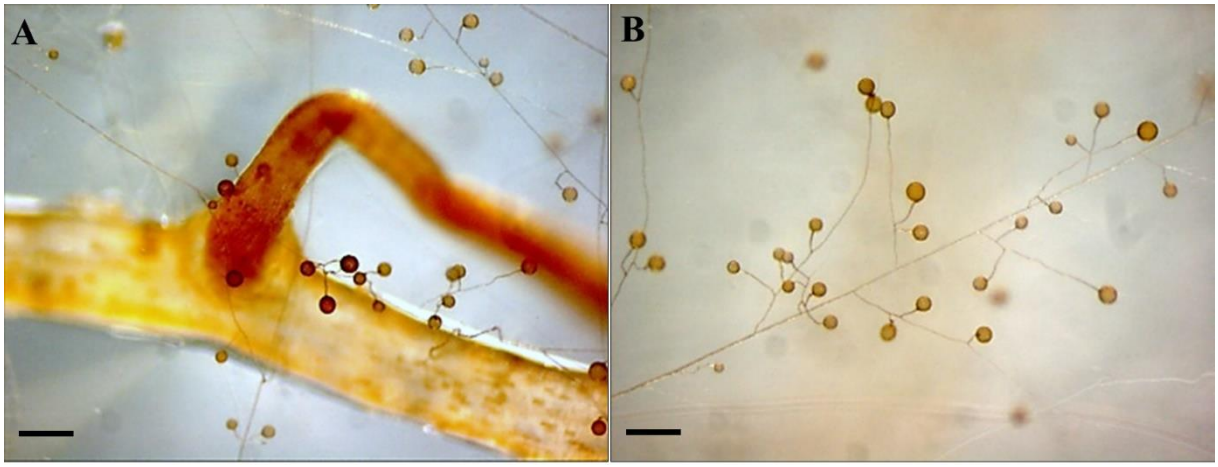


Fig. 15. *Rhizophagus irregularis* MUCL 41833 in root organ culture. (A) Ri T-DNA transformed carrot roots associated to the AM fungus (scale bar 120 µm) and (B) spores produced in the extraradical mycelium (ERM) (scale bar 120 µm). With permission from GINCO (Glomeromycota *in vitro* Collection).

R. irregularis was maintained *in vitro* by sub-cultivation at regular intervals (each three weeks) on carrot (*Daucus carota* L.) roots clone DC2 in 90 mm diam. bi-compartmented Petri plates with a vertical wall separating the root compartment (RC), containing the root and AM fungus, from the hyphal compartment (HC) in which only the fungus was allowed to grow (St Arnaud et al. 1996). The RC was filled with 25 ml Modified Strullu-Romand (MSR) medium (**see composition in Annex I**) and the HC received MSR medium without sucrose and vitamins. In both compartments, the MSR medium was solidified with 3 g L^{-1} Phytagel (Sigma-Aldrich, Germany) and pH adjusted to 5.5 before sterilization (121°C for 15 min) (St Arnaud et al. 1996; Declerck et al. 1998). The Petri plates were incubated in the dark at 27°C until several thousand spores were produced in the HC.

Gigaspora sp. MUCL 52331 was selected for its ability to grow under *in vitro* conditions associated with ROC. Recently, this strain was previously used to evaluate HHM in the

presence or absence of diesel (Garcés-Ruiz et al. 2021). Likely this *Gigasporaceae* specie could be less adapted to crop soils and then more associated to non-tillage soils (Orrù et al. 2021), sand dunes (Stürmer et al. 2018) and prairie grasslands (Ji et al. 2013). But only few information are available on the impact of agricultural practices on members of *Gigasporaceae* family. ***Gigaspora* sp.** (Gerdemann and Trappe) MUCL 52331 was isolated from a winter crop of rye (*Secale cereal* L.) in the center for Environmental Farming Systems in North Carolina (USA) (**Figure 16**) (Lalaymia et al. 2014). Species belonging to the *Gigasporaceae* family are more prevalent in stable ecosystems where inter- and intraspecific competition is high for resources, and somatic growth is favored over reproduction (K-strategist) (de Souza et al. 2005).

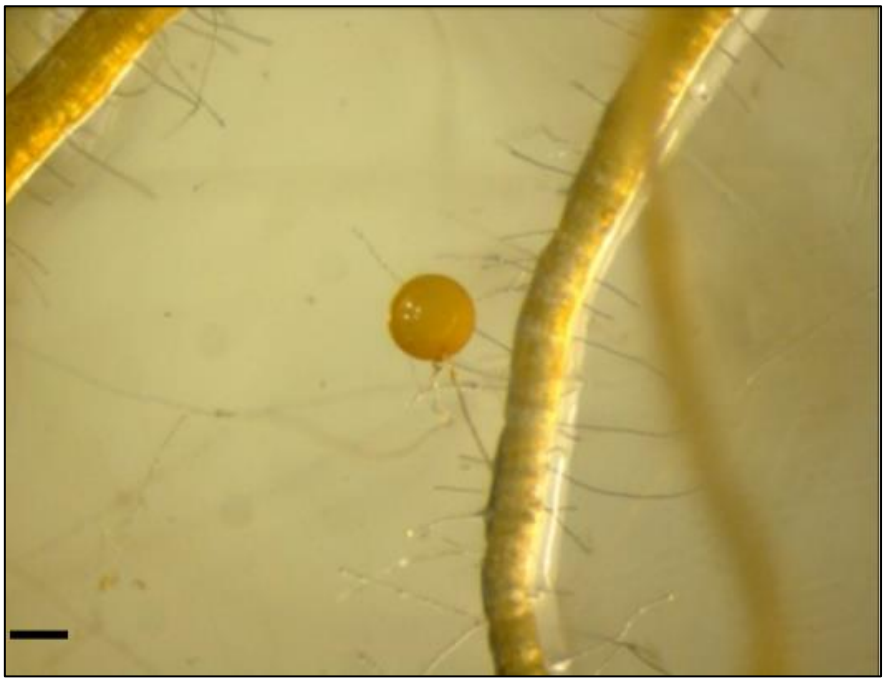


Fig. 16. *Gigaspora* sp. MUCL 52331 associated to Ri T-DNA transformed chicory roots (scale bar 100 μm). With permission from GINCO (Glomeromycota *in vitro* Collection).

This AM fungal strain was maintained *in vitro* by sub-cultivation at regular intervals (each three months) on chicory (*Cichorium intybus* L.) roots clone A4NH (Fontaine et al. 2004; Cranenbrouck et al. 2005) in 135 mm diam. mono-compartmented Petri plates containing 100 ml of MSR medium and incubated under conditions like *R. irregularis* (see above) (Declerck et al. 1998).

IV.1.2. Host plants

Maize (*Zea mays* L.) var. Troizi (Caussade®) seeds were received from "Caussade Semences" (www.caussade-semences.com, France). Maize is an important cereal crop of the grass family *Poaceae* adapted to different ecological systems (Scott and Emery 2016). This plant was selected for its fast growth, extensive root production and high capacity to associate with AM fungi under controlled greenhouse conditions.

Medic (*Medicago truncatula* L.), cv. Jemalong A17 seeds were received from the South Australian Research and Development Institute (SARDI, Australia). Medic is a small annual plant of the family *Fabaceae*, with a short life cycle of two months. This plant has become particularly well studied as a model organism for understanding the physiology of leguminous plants, including the root colonization processes by AM fungi (Goss et al. 2017).

Maize and medic were used for mass-production of *R. irregularis* under greenhouse conditions as follows: first the AM fungus was sub-cultured with carrot roots *in vitro*. It was then added to sterilized (121 °C for 15 min) lava stone substrate (DCM, Belgium) in association with seeds surface-disinfected by immersion in bleach (8% active chloride) for 15 min and rinsed 3 times with sterilized (121 °C for 15 min) deionized water for 10 minutes (Dupré de Boulois et al. 2006).

Potato (*Solanum tuberosum* L.) var. Bintje plantlets were provided by the Station de Haute Belgique in Libramont, Belgium. This variety was used because it is one of the main potato varieties cultivated in Belgium on a surface of 6830 ha in 2020 (Filière wallonne de la pomme de terre 2021). Remarkably, potato is a globally important crop plant producing high yields of nutritionally valuable food in the form of tubers. It has been the focus of substantial studies because of its use both as a staple food crop and as a potentially significant source of compounds of interest (Millam 2006).

Potato plantlets were micro-propagated every five weeks by placing nodal cuttings in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box) (Voets et al. 2005), filled with 50 ml of 4.41 g L^{-1} sterilized (121°C for 15 min) Murashige and Skoog medium (**see composition in Annex II**), supplemented with 20 g L^{-1} sucrose, 3 g L^{-1} Gelrite™ and adjusted to pH 5.5. Plantlets were kept in a growth chamber (Snijders Scientific B.V, Netherlands) at 20/15°C (day/night) and illuminated for 16 h d^{-1} under a photosynthetic photon flux of 150 µmol m^{-2} s^{-1}.

IV.2. Pesticide preparation

IV.2.1. Fungicide and herbicide preparation for *in vitro* experiments

The active ingredients (a.i.) of four fungicides were selected: azoxystrobin and flutolanil that disrupt respiration, pencycuron that disrupt cytoskeleton and motor proteins and fenpropimorph that affect the sterol biosynthesis in membranes (Fungicide Resistance Action Committee 2022).

The a.i. were supplied by Sigma-Aldrich, Inc. [Darmstadt, Germany]. The concentrations of pesticides used in the *in vitro* experiments were established on values used by Moorman (1986), Zocco et al. (2011) and Buysens et al. (2015). Last authors calculated the concentrations of pesticides hypothesizing that bulk soil density is 1.5 g cm^{-3}, that the fungicides penetrate down to 20 cm under the soil layer and that potato tubers are planted at a density of 2000 kg ha^{-1}. These calculated values may vary according to the agricultural practices recommended for each crop, commercial formulation or geographical area (**Table 6**).

Table 6. Recommended field dose and concentrations of pesticides estimated in the *in vitro* experiments.

Pesticide	**Glyphosate**	**Azoxystrobin**	**Flutolanil**	**Fenpropimorph**	**Pencycuron**
Formulations	Roundup	Amistar	Monarch	Corbel	Monceren
Crop	Soybean	Potato		Wheat	Potato
Pest	Weed	*Rh. solani*		Powdery mildew	*Rh. solani*
Field dose (a.i.)	1080 g ha^{-1}	1500 g ha^{-1}	184 g ha^{-1}	750 g ha^{-1}	500 g ha^{-1}
in vitro concentration estimated (a.i.)	0.8 mg L^{-1}	0.75 mg L^{-1}	0.09 mg L^{-1}	0.34 mg L^{-1}	0.25 mg L^{-1}

Noticeably, these values should not be extrapolated to represent the toxic effect of fungicides in the field because under field conditions, soil microbial and chemical activities would influence the effects of pesticides.

Each fungicide was dissolved in a solution of acetone (5 ml L^{-1} of MSR medium – Zocco et al. 2008) and added to a bottle containing 50 ml sterilized (121°C for 15 min) MSR medium. Indeed, at this concentration, no impact of acetone on the fungus has been reported (Zocco et al. 2008). In addition, the acetone evaporates once added in the hot MSR medium as reported by Zohrehvand (2005). The concentration of fungicides (0.02 and 2 mg L^{-1}) was adjusted in the MSR medium (Zocco et al. 2008). The bottles contained a stirring magnet to avoid solidification and to obtain a homogeneous concentration of the different a.i.

One herbicide was selected: glyphosate that impacts cellular metabolism by inhibition of the enolpyruvyl shikimate phosphate synthase (Herbicide Resistance Action Committee 2022). The a.i. was supplied by Sigma-Aldrich, Inc. [Buchs Switzerland]. Glyphosate was adjusted to increasing concentrations (0.02, 0.2 and 2 mg L^{-1}) by dissolution in deionized water (5 ml L^{-1} of MSR medium) and added to bottles containing 50 ml sterilized (121°C for 15 min) MSR medium.

IV.2.2. Azoxystrobin preparation for the semi-hydroponic circulatory system for the greenhouse experiment

In **Chapter 4,** the short-term depletion of Pi in the modified Hoagland solution of potato plants colonized or not by *R. irregularis* MUCL 41833 in presence or absence of the fungicide azoxystrobin was evaluated. A circulatory semi-hydroponic cultivation system was performed under greenhouse conditions.

The substrate used: perlite (KNOUF- GMBH, Germany) was sieved at 1 mm diam., rinsed with deionized water and dried in an oven for 48 h at 50°C (Garcés-Ruiz et al. 2017), before mixing it with fungicides. For this, 32 g of perlite was soaked in 32 mL of acetone containing 0.56 mg L^{-1} azoxystrobin (Sigma–Aldrich, Inc., Darmstadt, Germany) to obtain 1.17 mg of fungicide per gram of perlite (i.e., 37.5 mg per container, the azoxystrobin [AZO] treatment), a concentration per plant relative to the field dose recommended against *Rh. solani* in potato cropping systems in southern Belgium before plantation (i.e., 1500 g a.i. ha^{-1} Amistar) and considering a population of 40 000 plants ha^{-1} (Phytoweb 2021) recommended against *Rh. solani* in potato cropping systems.

Calculation:

According to Phytoweb, against *Rh. solani*, the soil is treated with Amistar at a concentration of 6 L ha^{-1} (once per crop) and this product contains 250 g L^{-1} of azoxystrobin.

6 L * 250 g = 1500 g ha^{-1} = 0.15 g mg/m²= 150 mg/m²

For the Bintje variety, we consider 40 000 plants, or 4 plants mg/m².

=> 150 mg/m² / 4 plants = 37.5 mg $plant^{-1}$

37.5 mg in a pot = 37.5 mg / 32 g of perlite = 1.17 mg g^{-1} of perlite.

IV.3. Experimental set-ups

In general, screening the impact of fungicides on AM fungi under *in vitro* conditions offers several advantages among which are the easy non-destructive observations of the ERM and sporulation dynamics, and the microbial-free experimental conditions avoiding any confounding effects with unwanted microorganisms (Fortin et al. 2002; Buysens et al. 2015). However, it is obvious that *in vitro* cultivation of AM fungi only partially reflects field situations. Microbes as well as chemical factors may degrade fungicides and reduce their activity with time. The transfer of nutrients, especially phosphorus is a key part of symbiosis between plants and AM fungi. To complement the *in vitro* studies, a circulatory semi-hydroponic cultivation system adapted to potato plants was set up to monitor the short-term depletion of Pi using modified Hoagland solution. The circulatory semi-hydroponic cultivation system is an adequate method for measuring Pi depletion in a nutrient solution and by corollary Pi uptake/immobilization by the plant-fungal associates. Because the measurements are non-destructive, the time course of Pi uptake can be monitored at different time points or at different phenological development steps without disturbing the growth of the plant and its fungal associate. Indeed nowadays, the contribution of AM fungi to P accumulation in plants is most often evaluated destructively (i.e., at harvest), thus by quantification of P at a single time point (Calonne-Salmon et al. 2018).

Root organ cultures (ROC) of AM fungi facilitate the study of environmental, nutritional, and physiological effects on fungal growth and spore production (Fortin et al. 2002). Indeed, the establishment of ROC *in vitro* have been used to investigate the impact of different fungicides on root colonization (Wan et al. 1998; Campagnac et al. 2008), spore production and mycelium development (Gong et al. 2014; Zocco et al. 2008; Campagnac et al. 2009; Cardenas-Flores et al. 2011), oxidative stress (Campagnac et al. 2008; González-Guerrero et al. 2010) and sterol biosynthesis (Oger et al. 2009; Calonne et al. 2012) of AM fungi.

IV.3.1. The monocompartmented root organ culture system for studying the hyphal healing mechanism events

The mono-compartmented ROC system was used to analyze the HHM of *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331 in the presence of the pesticides described above. This system consists of a Petri plate (135-mm diam.) with a single compartment containing 100 ml Modified Strullu–Romand (MSR) medium solidified in a slope (~10°) (**Figure 17**) (Declerck et al. 1998). In this system, the root organ (e.g., *Daucus carota* and *Cichorium intybus*) and the ERM grow into the same environment (Cranenbrouck et al., 2005). We used the mono-compartmented system for the culture of *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331 because it has been found that these strains produce an extensive ERM after six weeks and three months of incubation under inverted position at 27°C inside a growth chamber under dark conditions.

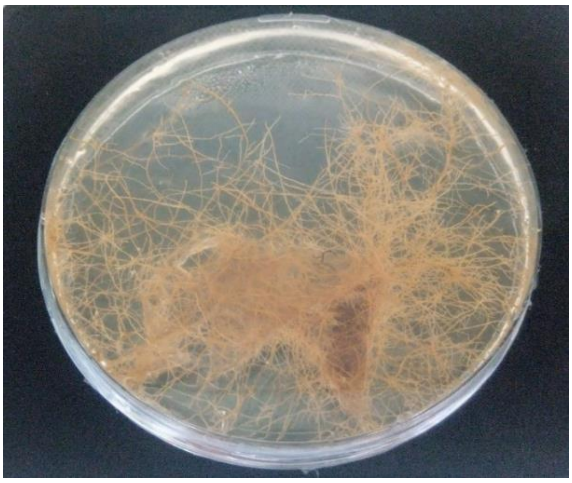


Fig. 17. The mono-compartmented ROC system used to analyze the hyphal healing mechanism (HHM) of *R. irregularis* MUCL 41833 and *Gigaspora* sp. associated to root organ of *Cichorium intybus*.

IV.3.2. The quadri-compartmented root organ culture system system or studying the anastomosis in extraradical mycelium

The quadri-compartmental ROC system was developed to investigate the capacity of *R. irregularis* MUCL 41833 to form anastomoses within and between extraradical mycelial networks of the same isolate under exposure of the fungicides described above. *In vitro* cultures of *R. irregularis* MUCL 41833 were established in rectangular culture plates comprising 4 adjoining compartments (whole plate 12.4 × 8.5 cm; each compartment 3.1 × 8.5 cm, quadri-PERM, Greiner Bio- One (Kremsmünster Austria)), but only three were used, two lateral compartments (LC) on both sides of one mycelium central compartment (CC) separated by plastic walls (**Fig. 18**) (Voets et al. 2006).

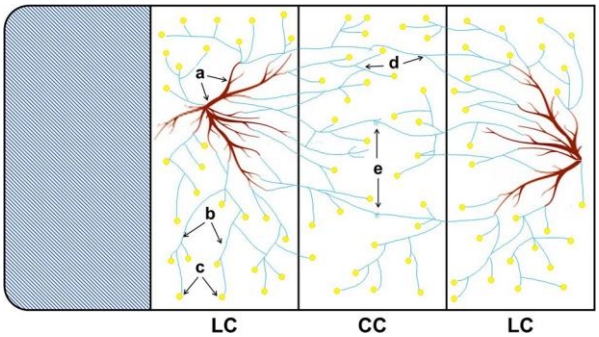


Fig. 18. Schematic representation of the quadri-compartmental culture system (whole plate 12.4 × 8.5 cm; each compartment 3.1 × 8.5 cm). A carrot root organ culture (ROC) (**a**) with mycelium (**b**) and spores (**c**) of *R. irregularis* MUCL 41833 was placed on the MSR medium in two lateral culture compartments (LC). After 4–6 weeks, the two LCs were covered with a dense extraradical mycelium (ERM) (**b**, **c**). Then, the central compartment (CC) was supplemented with MSR medium without fungicide ($MSR^{control}$) or ($MSR^{acetone}$) or two concentrations (0.02 and 2 mg L^{-1}) of azoxystrobin, pencycuron, flutolanil, and fenpropimorph. After a period of 15 days, the CC was covered with ERM. Finally, the anastomosis formation within network (**d**) or between networks (**e**) and ERM growth parameters were assessed in the CC.

IV.3.3. The circulatory semi-hydroponic cultivation system

A circulatory semi-hydroponic cultivation system was set up to monitor the short-term depletion of Pi in the modified Hoagland solution of potato plants colonized or not by *R. irregularis* MUCL 41833 in presence or absence of the fungicide azoxystrobin. This system is presented in **Figure 19**.

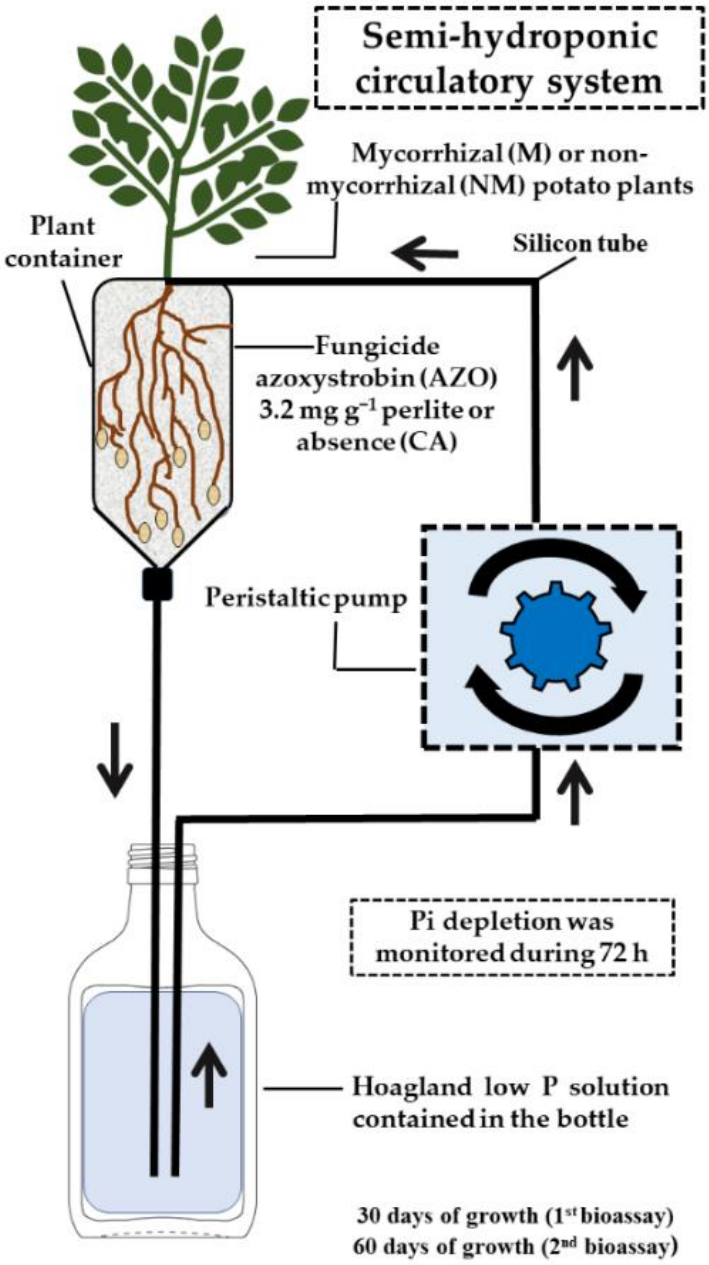


Fig. 19. Schematic representation of the circulatory semi-hydroponic cultivation system. The arrows represent the flow direction of the Hoagland-low P solution in the system.

This system uses a peristaltic pump (Gilson's Minipuls Evolution, France) linked to a bottle containing the Hoagland-low P solution, via cleaned (in sodium hypochlorite solution 50% overnight) 3 mm inner diameter silicon tubes (VWR, United States). The solution is thus pumped to the container of the potato plants. The plant containers consist of an inverted 75 mm diameter wash bottles (500 ml - VWR, USA) cut at the base. A mesh of 100 µm size pore

(Prosep B.V.B.A., Belgium) was glued on the top of the bottle to avoid losses of the substrate and to prevent roots from growing outside the bottle. Finally, the bottom of the containers is similarly connected to another silicon tube and the solution percolates through the plantlets and returns to the individual bottles. Each container is connected to a single bottle in a closed system.

The circulatory semi-hydroponic cultivation system was first used to evaluate the short-term phosphorus uptake rates in ectomycorrhizal and non-ectomycorrhizal *Pinus sylvestris* seedlings (Colpaert et al. 1999). Subsequently, this culture system was adapted to AM fungi associated to maize plants by Garcés-Ruiz et al. (2017). In the present study was focused on Pi which is a key element in the functionning of the symbiosis, but the plant system could be extended to analyze the uptake dynamics of other macro and micro-nutrients from their depletion in a determined nutrient solution.

IV.4. Measurement of root colonization percentage

AM structures within the roots are usually hidden by the natural pigments (i.e. tannins, polyphenols) and contents of root cells (Vierheilig et al. 2005). To evaluate root colonization by AM fungi it is thus necessary first to clear the roots. Roots clearing aims to soften the cells wall, empty cortical cells from their cytoplasm, removes tannins to improve the penetration of ink within the cells. Clearing is done by bathing the roots in KOH 10% for 45 minutes at 60°C. The roots are then washed free from the KOH and acidified with HCl 1%. After a novel rinsing with water, the roots are stained with a solution of blue ink (Parker, France) at 2% (Walker 2005). The ink will be preferentially fixed on fungal walls (composed of chitin and callose) than on the bleached walls of root cells.

Root colonization was assessed following the method of McGonigle et al. (1990). This method is based on the observation of a representative length of randomly selected root fragments, focusing on the presence of intra-radical structures, such as arbuscules, vesicles and hyphae. The presence of these structures is then used to infer the global rate of root system colonization. Fifteen 1-1.5 cm root fragments were mounted on slides and 3 slides per replicate were observed under a dissecting microscope (Olympus BH2- RFCA, Japan) at 10 x magnification. Three intersections per root fragment (meaning a total of 150 intersections per replicate) were observed for estimation of total root colonization (%TRC), percentage of arbuscules (%arbuscules) and percentage of spores/vesicles (%vesicles) (McGonigle et al. 1990) (**Figure 20**).

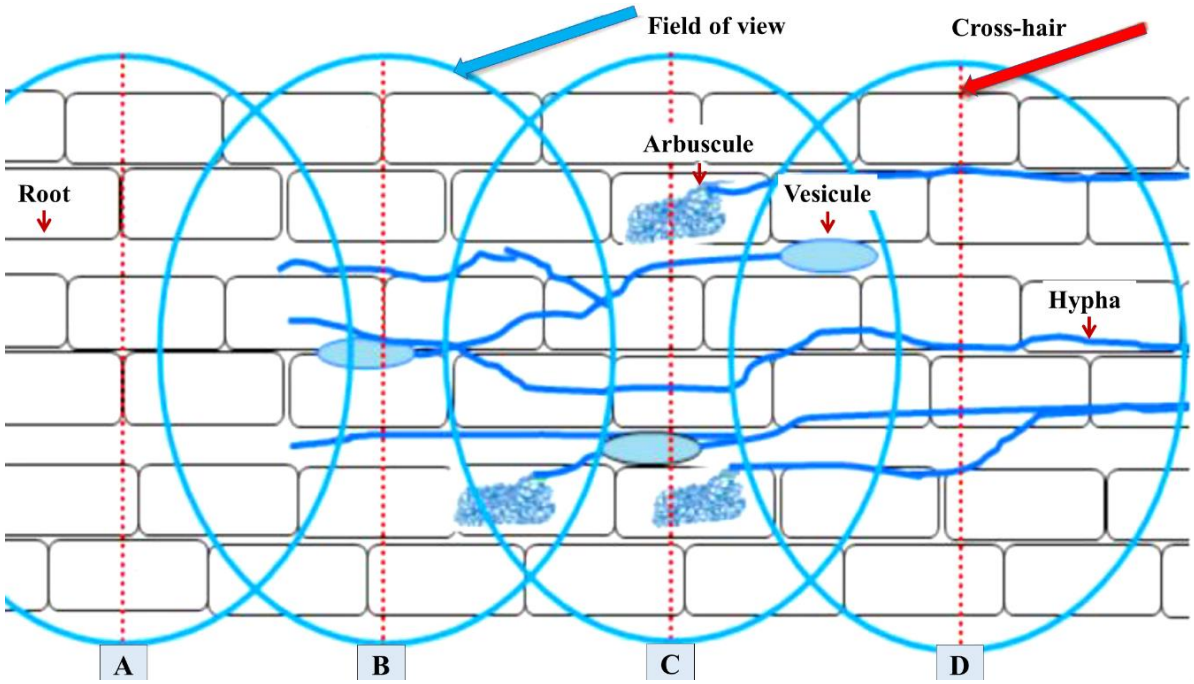


Fig. 20. Evaluation of the root colonization by AM fungi. (**A**) absence of AM fungus structures at cross-hair (negative-intersect); (**B**) observation of spores/vesicles or hyphae (presence of each single structure); (**C**) observation in the same intersect of spores/vesicles, arbuscules and hyphae (all structures); (**D**); when only hyphae were observed at the intersection (hyphae) (McGonigle et al. 1990).

Then, in the root systems of the mycorrhizal plants, the %arbuscules and %vesicles were calculated by dividing the number of intersections with the presence of arbuscules or spores/vesicles, respectively by the total number of intersections examined (i.e., %arbuscules and %vesicles = Total number of AM positive segments/Total number of segments examined × 100%). Finally, total colonization (%TRC) was calculated as the proportion of non-negative intersections (i.e., TRC % = Total number of AM positive segments/Total number of segments examined × 100%).

Research results: Part I

Impact of pesticides on the hyphal healing and anastomosis in two arbuscular mycorrhizal fungi with contrasting life history strategies

Preface

Arbuscular mycorrhizal (AM) fungi form an extensive network of extraradical hyphae in the soil that extends far beyond the root zone to acquire mineral nutrients and water for the host plant. This extraradical mycelium (ERM) network is favoured by the ability of hyphae to form anastomosis within the same hyphae and between different hyphae (often belonging to the same colony). Interestingly, this anastomosis process concerns non-perturbed hyphae as well as damaged hyphae. In this later case, the AM fungi develop a process of repair named hyphae healing mechanism (HHM). This mechanism has been fully described by de la Providencia et al. (2005). The HHM is crucial to maintain the integrity of ERM. For example, in agricultural soils, the ERM can be physically injured by practices such as soil ploughing and the subsequent HHM affected by fungicides application to control diseases. Nowadays, several studies have reported the impact of fungicides on root colonization and spore production of AM fungi but their impact on the process of HHM is totally unknown.

In **chapter I**, we designed a study to investigate the impact of four important fungicides (azoxystrobin, pencycuron, flutolanil and fenpropimorph) at two concentrations (0.02 and 2 mg L^{-1}) on the HHM process of two AM fungi with contrasting life history strategies (*Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833). Both AM fungi belong to the *Gigasporaceae* and *Glomeraceae*, respectively, of the two most representative AM families. After hyphae physical injury four events in the process of HHM (septum formation, emission, contact and fusion of growing hyphal tips) were monitored at regular intervals under a dissecting bright-field light microscope.

Chapter I

Fungicides with contrasting mode of action differentially affects hyphae healing mechanism in *Gigaspora* sp. and *Rhizophagus irregularis*

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Abstract

Fungicides are widely used in conventional agriculture to control fungal diseases but may also affect non-target microorganisms such as arbuscular mycorrhizal (AM) fungi. These root symbionts develop extended mycelial networks within the soil via mechanisms such as anastomosis that indistinctly concerns intact and damaged hyphae, the latter being named hyphal healing mechanism (HHM). The HHM differs between *Glomeraceae* and *Gigasporaceae*. However, the effects of fungicides on this mechanism in unknown. Here, the impact of azoxystrobin, pencycuron, flutolanil and fenpropimorph at 0.02 and 2 mg L^{-1} were tested *in vitro* on the HHM of *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833, and repair events visualized carefully under a dissecting bright-field light microscope. Azoxystrobin was the more detrimental for both AM fungi at 2 mg L^{-1}, while fenpropimorph impacted only *R. irregularis* (stimulating at low and inhibiting at high concentration). Conversely, flutolanil and pencycuron did not impact any of the two AMF fungi. The mechanisms involved remains to be elucidated, but perturbation in the still-to-be firmly demonstrated spitzenkörper or in sterols content as well as a process of hormesis are possible avenues that deserve to be explored in view of a rationale management of chemicals to control fungal pathogens without harming the beneficial AM fungi.

Keywords

Arbuscular mycorrhizal fungi, Extraradical mycelium, Hyphal healing mechanism, Fungicides, Growing hyphal tips.

INTRODUCTION

The extraradical mycelium (ERM) of arbuscular mycorrhizal (AM) fungi is fundamental in plant nutrition and maintenance of biological fertility in agroecosystems (Pepe et al. 2018; de Novais et al. 2019a). Keeping the integrity of this belowground network is thus essential, not only for the survival of these fungi but also for their manifold benefits to plants. Mechanisms such as anastomosis and healing are pivotal for the spread and maintenance/survival of AM fungal colonies (de la Providencia et al. 2005). Both mechanisms drastically differ between genera (de la Providencia et al. 2005; Voets et al. 2006) and could be affected by agricultural practices such as ploughing (Brito et al. 2012) or application of pesticides (de Novais et al. 2019b; Hage-Ahmed et al. 2019).

Anastomosis, that is the process of fusion between branches of the same or different hyphae to constitute a mycelial network (Kirk et al. 2008), has been abundantly reported in the fungal kingdom (Glass et al. 2000) and described in intact two-dimensional (Giovannetti et al. 2004) as well as three-dimensional (de la Providencia et al., 2005) ERM networks of AM fungi. It indistinctly concerns intact and damaged hyphae, the latter being described as a hyphal healing mechanism (HHM) (de la Providencia et al. 2007). The HHM has been described in four successive events. First, it begins with the formation of a septum near or in the apical zone on both sides of the injured hyphae, which prevents massive cytoplasmic/protoplasmic leakage into the surrounding environment. Secondly, one or several growing hyphal tips (GHTs) emerge from both extremities of the cut hyphae either protruding through the septum or emerging behind it. Thirdly, the GHTs elongate and grow towards each other and in most cases enter into contact. Fourthly, fusion is observed between GHTs with re-establishment of cytoplasmic/protoplasmic flow (de la Providencia et al. 2005). Importantly, the HHM differ between *Glomeraceae* and *Gigasporaceae*. Indeed, the HHM in *Glomeraceae* is oriented towards the reconnection of the affected area by linking several hyphae in relatively small vicinity or by recolonization of roots and substrate, by contrast to *Gigasporaceae* in which the HHM is nearly always oriented towards the re-establishment of hyphal integrity (de la Providencia et al. 2007). This suggests that both fungi have developed different strategies to grow and survive under adverse conditions (de la Providencia et al. 2005).

Fungicides are widely used in conventional agriculture to control fungal diseases. Unfortunately, these molecules may also affect soil plant-beneficial microorganisms such as AM fungi (Jin et al. 2013; Buysens et al. 2015). Their impact on these below-ground microorganisms have been investigated under field (e.g., Shalamuk et al. 2014; Rivera-Becerril et al. 2017), greenhouse (e.g., Jin et al. 2013; Channabasava et al. 2015; Rabab and Reda, 2019) and growth chamber (de Novais et al. 2019a) conditions as well as *in vitro* on root organs (e.g., Campagnac et al. 2008; Zocco et al. 2008) or whole plants (e.g., Zocco et al. 2011). *In vitro* cultivation systems offer several advantages, among which, the absence of any confounding effects caused by unwanted contaminants or environmental factors (e.g., soil physico-chemical parameters) and the easy non-destructive observations of growth and development of fungi. Spore germination (Chiocchio et al. 2000; Zocco et al. 2008; Buysens et al. 2015), root colonization (Campagnac et al. 2008, 2009; Calonne et al. 2010, 2012), anastomosis formation (Cardenas-Flores et al. 2011; de Novais et al. 2019a, 2019b), sterol biosynthesis pathway (Campagnac et al. 2010; Calonne et al., 2012) and transport of nutrients (e.g., phosphorus) from fungus to plant (Zocco et al. 2011) have been investigated *in vitro* in presence of different types of fungicides. Results differed significantly with fungicide and dose of application. However, no study has reported the effects of fungicides on the HHM and thus on the ability of hyphae to maintain integrity following physical disturbance.

Azoxystrobin, pencycuron, flutolanil and fenpropimorph are amongst the most frequently used fungicides to control soil fungal diseases. Azoxystrobin is a systemic fungicide that belongs to the class of methoxyacrylates, which are derived from the naturally occurring strobilurins. It is the most widely sold fungicide worldwide (Zhang et al. 2019), used against several fungal diseases of many edible crops and ornamental plants. It exhibits its fungicidal activity by binding to the quinol oxidation (Q_o) site of cytochrome b to inhibit mitochondrial respiration in fungal species from the Ascomycota, Basidiomycota and Deuteromycota and fungal-like species from the Oomycota (Bartlett et al. 2002; Feng et al. 2020). Pencycuron is a phenylurea fungicide of contact, which is highly specific to *Rhizoctonia solani*, inhibiting mycelial growth by blocking cell division and destroying the cytoskeleton of the microtubules during mitosis (Young et al. 2012). Flutolanil is a systemic phenyl benzamide fungicide, used against diseases caused by Basidiomycota in crop plants (Zhao et al. 2019). It mainly inhibits the hyphal growth and infection formation, and strongly reduces the mycelial O_2 consumption of *R. solani* as well as the activity of succinate dehydrogenase complex (Complex II) in mitochondria (Mol et al. 2019). Finally, fenpropimorph is a morpholine of broad-spectrum considered as a sterol biosynthesis inhibitor. It specifically inhibits at low concentrations the sterol $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, and additionally, when it is used at higher concentrations, inhibits Δ^{14}-sterol reductase (Marcireau et al. 1990). This fungicide is mainly applied in cereals to control *Blumeria* (powdery mildew) and *Puccinia* (cereal rust) species (Stenzel and Vors 2019).

A number of studies under strict *in vitro* culture conditions have investigated the effects of these fungicides on AM fungi. Azoxystrobin and its formulation Amistar did not impact spore germination and root colonization of potato associated with *R. irregularis* MUCL 41833 at threshold concentration (IC50 $\leq$ 0.1 mg L^{-1} a.i.) for the control of *R. solani*, while at 10 times this threshold, spores production and mycelium development were significantly affected (Buysens et al. 2015). In the same study, at threshold value for the control of *R. solani*, pencycuron and its formulation Monceren, did not affect spore germination and intra- or extraradical mycelium development (Buysens et al. 2015). Finally, flutolanil and its formulation Monarch at threshold value for the control of *R. solani* did not affect spores germination or extraradical mycelium development but decreased root colonization and arbuscules formation. In other studies, using root organ cultures (ROC) (Zocco et al. 2008; Campagnac et al. 2008, 2009, 2010; Oger et al. 2009) or whole plants (Zocco et al. 2011), the effects of fenpropimorph on the AM fungal symbiosis was demonstrated. Fenpropimorph presented a high toxicity with drastic sterols modifications in the host roots which was mirrored by a drastic reduction of root growth, root colonization and decrease of phosphorus transport, alkaline phosphatase and succinate dehydrogenase activities of the ERM (Zocco et al. 2011). All these studies suggested that fungicides may have undesirable effects on AM fungi, but none considered their effects on HHM.

The objective of this study was to investigate under *in vitro* culture conditions the impact of two different concentrations (0.02 and 2 mg L^{-1}) of azoxystrobin, pencycuron, flutolanil, and fenpropimorph on the HHM of two AM fungi (*Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833) belonging to phylogenetically distant families and thus having different life history strategies.

Materials and methods

Biological material

The AM fungi *Gigaspora* sp. (Gerdemann and Trappe) MUCL 52331 and *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] MUCL 41833 were supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). Both strains were maintained in association with Ri T-DNA transformed chicory (*Cichorium intybus* L.) roots on 135 mm diam. Petri plates containing 100 ml Modified Strullu–Romand (MSR) medium (Declerck et al. 1998).

Fungicide medium preparation

The active ingredients (a.i.) of four fungicides that disrupt respiration (azoxystrobin and flutolanil), cytoskeleton and motor proteins (pencycuron) and sterol biosynthesis in membranes (fenpropimorph) (Fungicide Resistance Action Committee 2020) were supplied by Sigma-Aldrich, Inc. [Darmstadt, Germany]. Each fungicide was dissolved in a solution of acetone (5 ml L^{-1} of MSR medium) and added to a bottle containing 50 ml sterilized (121°C for 15 min) MSR medium. The a.i. were added at a concentration of 0.02 and 2 mg L^{-1} MSR medium (Zocco et al., 2008). The bottles contained a stirring magnet to avoid solidification and obtain a homogeneous concentration of the different a.i. One bottle containing MSR medium without fungicides but added with acetone ($MSR^{acetone}$) and another with MSR medium alone ($MSR^{control}$) were used as controls.

Experimental design

The Petri plates were placed in a slope (~ 10°) during filling of the MSR medium so that medium was thinner at one side of the Petri plate at solidification. One chicory root piece was placed in the thicker side of the Petri plate and associated with one of the AM fungi. For *Gigaspora* sp., a 3 x 3 cm piece of gel containing mycelium and three spores from a six-month-old ROC was placed close to the root, while for *R. irregularis*, a 2.5 x 2.5 cm sliced piece of gel containing mycelium and approximately 500 spores from a two-month-old ROC was used. The Petri plates were incubated in an inverted position at 27°C in a growth chamber under dark conditions. After 12 and 6 weeks of growth for *Gigaspora* sp. and *R. irregularis*, respectively, Petri plates showing adequate extraradical mycelium development were selected. The selected hyphae (an average of 8 per plate) were growing on the surface of the MSR medium in the thinner part of the Petri plates and showing dense cytoplasmic/protoplasmic flow under a dissecting bright-field light microscope (Olympus SZ2-CTV, Japan) at 40 x magnification were selected for mechanical injury in each Petri plate. The HHM events were recorded in two Petri plates per treatment [i.e. each control treatment ($MSR^{treatment}$, $MSR^{Acetone}$) and fungicide concentration (0.02 and 2 mg L^{-1})] and per fungal strain (*Gigaspora* sp. and *R. irregularis*). Because the number of observations was important, the experiment was divided into two repetitions delayed in time for each tested fungicide. For each strain, one plate of each treatment (e.g., $MSR^{treatment}$, $MSR^{Acetone}$, 0.02 and 2 mg L^{-1} of one considered fungicide) was evaluated simultaneously. This was repeated once a week later.

In a plate, the selected hyphae were chosen at a reasonable distance from each other to avoid as much as possible interferences (i.e. hyphae contact from different cuts). Under horizontal laminar hood, hyphae were cut with a sterilized scalpel under the dissecting bright-field light microscope at 6.7 x to 40 x magnification. Immediately after cutting, 20 µl of MSR medium

without fungicides ($MSR^{control}$) or containing one of the two concentrations of fungicides or containing only acetone ($MSR^{acetone}$) was immediately added at the place of injury.

The fungicide treatments were labelled as follows: azoxystrobin$^{0.02mgL^{-1}}$, azoxystrobin$^{2mgL^{-1}}$, flutolanil$^{0.02mgL^{-1}}$, flutolanil$^{2mgL^{-1}}$, pencycuron$^{0.02mgL^{-1}}$, pencycuron$^{2mgL^{-1}}$, fenpropimorph$^{0.02mgL^{-1}}$ and fenpropimorph$^{2mgL^{-1}}$. The cut hyphae were observed cautiously at regular intervals under the dissecting bright-field light microscope at 100 x or 200 x magnification, first frequently during the first 60 min, then each hour until 16 h and finally after 24, 30, 36 and 48 h (**Figure 21**). This timeline was defined according to the observations of de la Providencia et al. 2005, 2007 in *Gigasporaceae* and *Glomeraceae* members growing under similar *in vitro* conditions. After 48 h, hyphae usually continue to circulate cytoplasm through the same hyphae or connected to other hyphae or nearby roots as in the case of *R. irregularis*. In the case of non-reconnection, the cytoplasm is transported in the opposite direction to the injury site (de la Providencia et al. 2005, 2007).

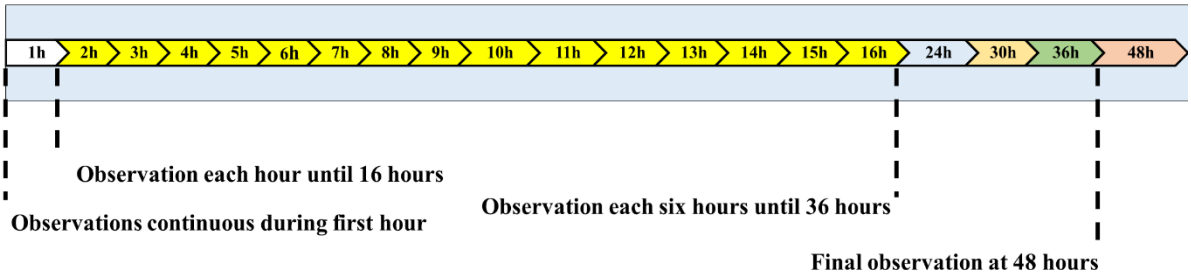

Fig. 21 Estimated monitoring time for hyphal healing mechanism (HHM) of arbuscular mycorrhizal fungi per cut.

The hyphae were visualized carefully under a dissecting bright-field light microscope (Olympus BH2–RFCA, Japan) at 40 x or 100 x magnification (**Fig**. **22A** and **22B**).

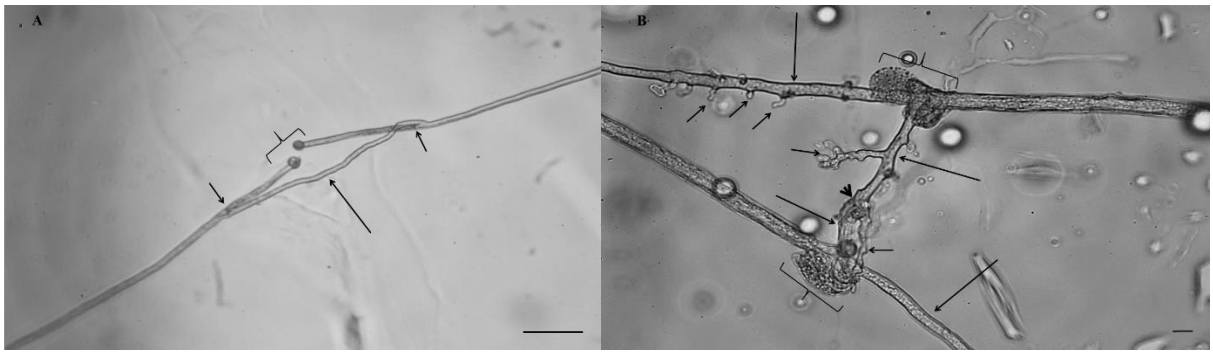

Fig. 22. Hyphal healing mechanism (HHM) of AM fungi in the control treatment (**A**) *Gigaspora* sp. MUCL 52331, the plugs were formed at both extremities in front of the hyphal injury (braces), two growing hyphal tips (GHTs) emerged at both extremities behind the septum (short arrows) and fused (large arrow) after the GHT contact with the re-establishment of the cytoplasmic/protoplasmic flow (scale bar 50 µm). (**B**) *Rhizophagus irregularis* MUCL 41833, the plugs were formed at both extremities in front of the hyphal injury (braces), two GHTs from each injury site emerged through the septum (large arrows) and one GHT of each side contacted and fused (arrowhead) with the re-establishment of the cytoplasmic/protoplasmic flow. Several hyphal branches (short arrows) developed from the GHTs (scale bar 10 µm) (pictures from M Calonne-Salmon, laboratory of Mycology, UCLouvain).

In addition, the cut hyphae were monitored cautiously by images captured with a digital camera (Canon EOS 60D) coupled to a compound bright-field light microscope (Olympus BH2–RFCA, Japan) at 100 x or 200 x magnification.

Data collection

Following hyphae cutting, four events in the HHM were monitored under microscopic observation: in each injured hypha (1) septum formation; (2) emission of growing hyphal tips (GHT) (3) contact of the GHTs and (4) fusion of the GHTs. These events were recorded as percentages in each treatment to perform an initial descriptive statistical analysis and then used in the Cox model (see below) considering the period from which the event was observed. Additionally, the number of GHTs and total number of hyphal branches coming from the GHTs were counted in the last point of the evaluation at 48 h.

The observed numbers are reported in **Figures 23** and **24**. The percentage of observed events was calculated from the total of injuried hyphae within each treatment and control group are reported in **Table 7** and for the $MSR^{control}$ group (result not shown). After 48 h, hyphae usually continue to circulate cytoplasm through the same hyphae (i.e. *Gigaspora* sp.) or connected to other hyphae or nearby roots (i.e. *R. irregularis*). In the case of non-reconnection, the cytoplasm was transferred toward the opposite direction to the injury site (de la Providencia et al. 2005, 2007).

Statistical analysis

To evaluate the impact of the fungicides on emission, contact and fusion of GHTs, each hyphal injury from all treatments, was considered. A Cox proportional hazard model for right and interval censored (time-to-event) data was fitted using R software version 4.0.0. The cox model was performed to analyze HMM events because it considers two important factors in the same model: the absence or presence of the event and the time elapsed since the start of the evaluation. Specifically, the *MIICD.coxph* function of the Multiple Imputation for Interval Censored Data R package was used (Delord, 2015). The non-observed events of the HHM after 48h were considered as right censored. More information on the time-to-event statistical modelling framework and more precisely on the Cox model can be found in Cox (1972) and George et al. (2014). The hazard ratio's (hr) reported in Tables 2 and S2 and defined as exp (β), where the β's are the coefficient estimates obtained from the fit of the Cox model, are interpreted as follows: an hazard ratio greater (respectively smaller) than one means that the event is more (respectively less) likely (i.e. the event occurs more (respectively less) often or is sooner (respectively later) observed) within the given treatment group compared to the control. In our analysis, $MSR^{acetone}$ is defined as the control group. For a seek of simplicity, **Table 8** only reports the estimates (obtained from the Cox model) for the treatment groups. The ones corresponding to MSR^{water} are reported in Table S1 since no significant difference was highlighted by the analysis between $MSR^{acetone}$ and MSR^{water}.

In addition, the number of GHTs and hyphal branches produced did not meet the assumption of normality**,** then these variables were subjected to Kruskal–Wallis non-parametric test followed by a post hoc Dunn multiple comparison test to validate significant difference between fungicides treatments and their respective control acetone using the software International Business Machines Corporation Statistical Package for the Social Sciences statistic 26 software. For all analyses, statistical significance was established at a 95% confidence level (i.e. when the p-value is less than 0.05).

Results

Hyphal healing mechanism in *Gigaspora sp.* and *R. irregularis* in the $MSR^{acetone}$ and $MSR^{control}$ treatments

Whatever the AM fungus, a loss of cytoplasmic/protoplasmic content was observed at both extremities of the cut hyphae (**Fig. 22A** and **22B**). A septum was formed and a change in color inside the hyphae (dark brown to brown) was noticed within the first 180 minutes. The mechanism of HHM slightly differed between both AM fungi, especially in the number of GHT produced. With *Gigaspora* sp., a low number of GHT (with rare hyphal branches) was noticed emerging behind both extremities of the injured hyphae (**Figure 22A**). In most of the cases the GHTs grew towards each other, reconnected and reestablished cytoplasmic/protoplasmic flow. In contrast, *R. irregularis* produced a high number of GHTs at both extremities growing through the septum and often bearing several hyphal branches. Hyphal re-growth appeared disorganized with a lower percentage of reconnections as compared with *Gigaspora* sp. Following healing, cytoplasmic/protoplasmic flow was re-established. Whatever the parameter (emission, contact and fusion of GHTs and number of GHTs and hyphal branches produced), no significant difference was highlighted between the $MSR^{acetone}$ and $MSR^{control}$ treatments (see data in supporting information), thus the impact of fungicides on the HHM was compared solely to the $MSR^{acetone}$ control treatment.

Impact of fungicides on the hyphal healing mechanism in *Gigaspora* sp. MUCL 52331

The impact of azoxystrobin, pencycuron, flutolanil and fenpropimorph at 0.02 and 2 mg L^{-1} was evaluated on the HHM in *Gigaspora* sp. MUCL 52331 (**Tables 7** and **8**). A significant effect of the $azoxystrobin^{2mgL-1}$ treatment was noticed for GHT emission ($hr = 0.134$, $P < 0.001$), contact ($hr = 0.064$, $P < 0.001$) and fusion ($hr = 0.044$, $P < 0.001$) (**Table 8**). All events were less often (or later in time) observed in presence of $azoxystrobin^{2mgL-1}$ as compared to the $MSR^{acetone}$ treatment. The number of GHTs ($P = 0.010$) and hyphal branches ($P = 0.004$) was significantly lower in the $azoxystrobin^{2mgL-1}$ treatment as compared to the $MSR^{acetone}$ treatment (**Figure 23**). Within a period of approximately 360 minutes following injury in the $azoxystrobin^{2mgL-1}$ treatment, the hyphae that did not produce GHTs showed retraction of cytoplasm in the opposite direction to the tip followed by formation of numerous septa inside the hyphae (result not shown). Although the percentage of all HHM observed events was lower in the $azoxystrobin^{0.2mgL-1}$ compared to the ones in the $MSR^{acetone}$ treatment, no significant effect was revealed from the Cox Model (**Table 8**). The emission, contact and fusion of GHTs (**Tables 7** and **8**) as well as number of GHTs and hyphal branches were not affected at both concentrations of pencycuron, flutolanil and fenpropimorph (**Figure 23**) as compared to the $MSR^{acetone}$ treatment.

Table 7. Data percentages calculated from the total of observations in each treatment of hyphal branches production, growing hyphal tips (GHT) emission, contact and fusion of the hyphal healing mechanism (HHM) of *Gigaspora sp.* MUCL 52331 and *Rhizophagus irregularis* MUCL 41833 in absence (control acetone) or in presence of increasing concentrations of azoxystrobin, pencycuron, flutolanil and fenpropimorph (0.02 and 2 mg L^{-1}).

	***Gigaspora* sp. MUCL 52331**			***Rhizophagus irregularis* MUCL 41833**		
	Control Acetone	**0.02 mg L^{-1}**	**2 mg L^{-1}**	**Control Acetone**	**0.02 mg L^{-1}**	**2 mg L^{-1}**
Azoxystrobin						
Hyphal branches (%)	94.4	86.7	35	73.7	66.7	50
GHT emission (%)	94.4	86.7	30	100	88.9	87.5
GHT contact (%)	88.9	86.7	15	73.7	66.7	37.5
GHT fusion (%)	88.9	73.3	10	68.4	55.5	31.2
Number of injured hyphae (n)	18	15	20	19	18	16
Pencycuron						
Hyphal branches (%)	81.2	81.8	64.3	94.4	88.9	86.7
GHT emission (%)	87.5	81.8	92.8	94.7	100	100
GHT contact (%)	81.2	81.8	85.7	89.5	83.3	73.3
GHT fusion (%)	81.2	81.8	71.4	73.7	72.2	60
Number of injured hyphae (n)	16	12	14	19	18	15
Flutolanil						
Hyphal branches (%)	72.2	83.3	81.2	94.7	100	100
GHT emission (%)	94.4	94.4	93.7	100	100	100
GHT contact (%)	83.3	77.7	93.7	89.5	90	88.9
GHT fusion (%)	83.3	72.2	75	73.7	75	77.8
Number of injured hyphae (n)	18	18	16	19	20	18
Fenpropimorph						
Hyphal branches (%)	87.5	86.7	56.2	85	86.7	70.6
GHT emission (%)	100	100	93.7	90	100	88.2
GHT contact (%)	100	93.3	87.5	85	80	35.3
GHT fusion (%)	87.5	73.3	75	70	73.3	29.4
Number of injured hyphae (n)	16	15	16	19	18	15

Table 8. Results of Cox proportional hazards regression analysis on growing hyphal tips (GHT) emission, contact and fusion events of the hyphal healing mechanism (HHM) for *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833 in presence of increasing concentrations of azoxystrobin, pencycuron, flutolanil and fenpropimorph (0.02 and 2 mg L^{-1}).

		***Gigaspora* sp. MUCL 52331**			***Rhizophagus irregularis* MUCL 41833**		
	Fungicide concentration (mg L^{-1})	**β**	**Hazard ratio**	**P - Value**	**β**	**Hazard ratio**	**P - Value**
Azoxystrobin							
GHT emission	0.02	-0.392	0.676	0.296	-0.457	0.633	0.187
	2	-2.009	0.134	**<0.001**	-0.685	0.504	0.056
GHT contact	0.02	-0.424	0.655	0.259	-0.200	0.818	0.613
	2	-2.444	0.064	**<0.001**	-1.147	0.317	**0.020**
GHT fusion	0.02	-0.568	0.567	0.140	-0.345	0.708	0.416
	2	-3.129	0.044	**<0.001**	-1.224	0.294	**0.021**
Pencycuron							
GHT emission	0.02	0.083	1.086	0.847	0.484	1.623	0.164
	2	0.124	1.133	0.748	0.520	1.682	0.154
GHT contact	0.02	0.243	1.275	0.578	0.114	1.120	0.749
	2	-0.050	0.951	0.901	-0.135	0.873	0.728
GHT fusion	0.02	-0.092	0.912	0.840	0.258	1.295	0.506
	2	-0.311	0.733	0.464	-0.151	0.860	0.725
Flutolanil							
GHT emission	0.02	0.628	1.873	0.073	NA	NA	NA
	2	0.403	1.496	0.267	NA	NA	NA
GHT contact	0.02	0.178	1.195	0.633	0.075	1.078	0.825
	2	0.539	1.714	0.144	0.002	1.002	0.995
GHT fusion	0.02	0.049	1.050	0.899	0.139	1.149	0.709
	2	-0.222	0.801	0.569	0.120	1.127	0.752
Fenpropimorph							
GHT emission	0.02	0.139	1.149	0.711	1.275	3.580	**<0.001**
	2	-0.415	0.661	0.282	-0.345	0.708	0.334
GHT contact	0.02	-0.475	0.622	0.201	0.805	2.238	**0.033**
	2	-0.414	0.661	0.263	-1.294	0.274	**0.007**
GHT fusion	0.02	-0.538	0.584	0.185	0.638	1.893	0.109
	2	-0.247	0.781	0.533	-1.224	0.294	**0.019**

Values were considered significant at $P < 0.05$ compared to the acetone control treatment.
β = Regression coefficient.
Hazard ratio = Exp (β).
NA = Not applicable.

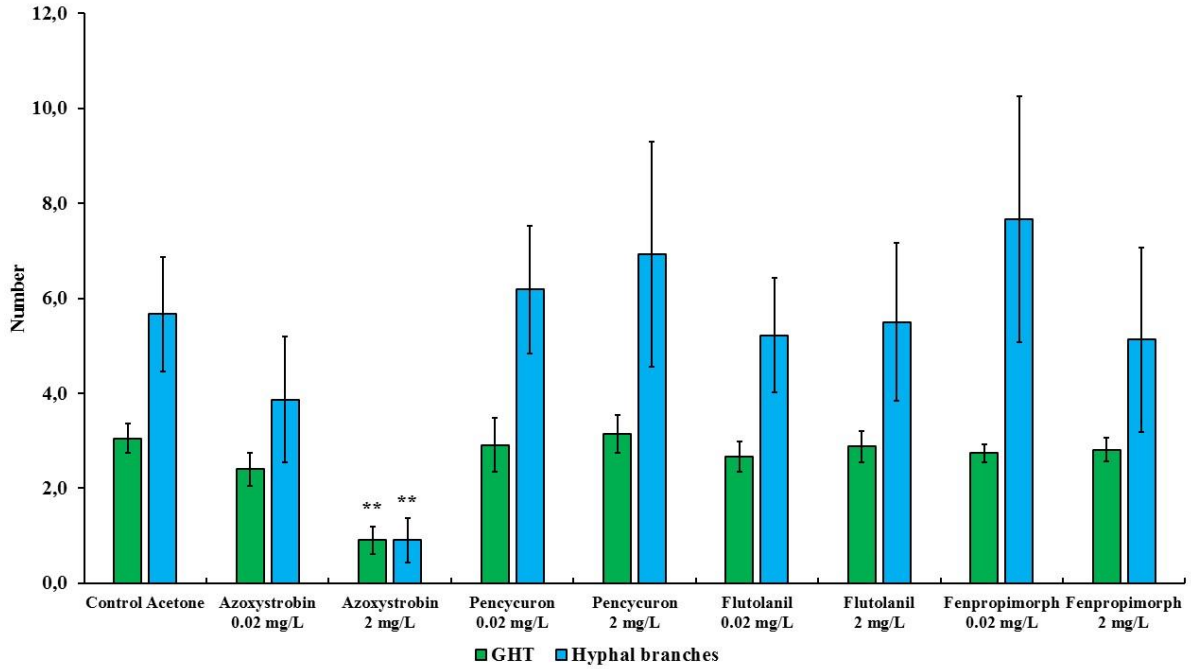

Fig. 23. Number of growing hyphal tips (GHT) and their hyphal branches produced in the hyphal healing mechanism (HHM) of *Gigaspora* sp. MUCL 52331 in absence (control acetone) or in presence of increasing concentrations of azoxystrobin, pencycuron, flutolanil and fenpropimorph (0.02 and 2 mg L^{-1}). Hyphal branches were numbered 48h after hyphal physical injury. Data are presented means ± standard error (two Petri plates per treatment with several injuries in each plate) and were analyzed by a Kruskal-Wallis test followed by a Dunn multiple comparison post-hoc test d to validate significant difference between fungicides treatments and their respective control acetone: *, ** and *** indicate P < 0.05; 0.01 and 0.001, respectively.

Impact of fungicides on hyphal healing mechanism in *Rhizophagus irregularis* MUCL 41833

The impact of azoxystrobin, pencycuron, flutolanil and fenpropimorph at 0.02 and 2 mg L^{-1} was evaluated on the HHM of *R. irregularis* MUCL 41833 (**Tables 7** and **8**). The results obtained from the Cox model revealed that the azoxystrobin^{2mgL-1} treatment had a significant impact on GHTs contact and fusion as the hazard ratio's were significantly less than 1 (hr = 0.317, P = 0.020 and hr = 0.294, P = 0.021, respectively) meaning that both events were less frequently observed within the azoxystrobin^{2mgL-1} treatment as compared to the MSR^{acetone} one, while no significant effect of the azoxystrobin^{2mgL-1} on GHT emission was found. Although a decreased trend in the percentage of observed GHTs emission, contact, and fusion events was noted in the azoxystrobin$^{0.02mgL-1}$ treatment, no significant difference was reported by our analysis (**Tables 7** and **8**). The number of GHTs and hyphal branches did not differ at both concentrations of azoxystrobin compared to the MSR^{acetone} treatment (**Figure 24**). After the formation of GHT, the HHM process was inhibited in the azoxystrobin^{2mgL-1} treatment. At the end of the evaluation, a limited growth of GHTs was observed and no septa was noticed in the injured hyphae of the azoxystrobin^{2mgL-1} treatment with the exception of a septum in the area of the lesion.

The GHTs emission, contact, and fusion events were not significantly affected by both concentrations of pencycuron and flutolanil as compared to the MSR^{acetone} treatment (**Tables 7** and **8**). In the case of flutolanil, the Cox proportional hazard model was not performed in the GHT emission event because GHTs formation was observed in all the injuries (**Table 7**).

Moreover, the number of GHTs and hyphal branches did not differ at both concentrations treatments (pencycuron and flutolanil) as compared to the MSR^{acetone} treatment (**Figure 24**).

Finally, the results of the Cox model showed that the fenpropimorph^{2mgL-1} treatment reduced significantly the percentage of observed GHTs contact (hr = 0.274, P = 0.007) and fusion (hr = 0.294, P = 0.019) (**Table 8**). In presence of fenpropimorph^{2mgL-1}, the number of GHTs (P = 0.015) and the number of hyphal branches (P = 0.039) was significantly lower as compared to the MSR^{acetone} treatment (**Figure 24**). After the formation of GHTs, the HHM process was inhibited in the fenpropimorph^{2mgL-1} treatment. At the end of the evaluation, a limited growth of GHTs was observed and no septa was noticed in the injured hyphae of fenpropimorph^{2mgL-1} treatment with the exception of a septum in the area of the lesion. On the other hand, in the fenpropimorph$^{0.02mgL-1}$ treatment, GHTs emission (hr = 3.580, P < 0.001) and contact (hr = 2.238, P = 0.033) were stimulated significantly, showing this event more frequently than in the MSR^{acetone} treatment (**Table 8**).

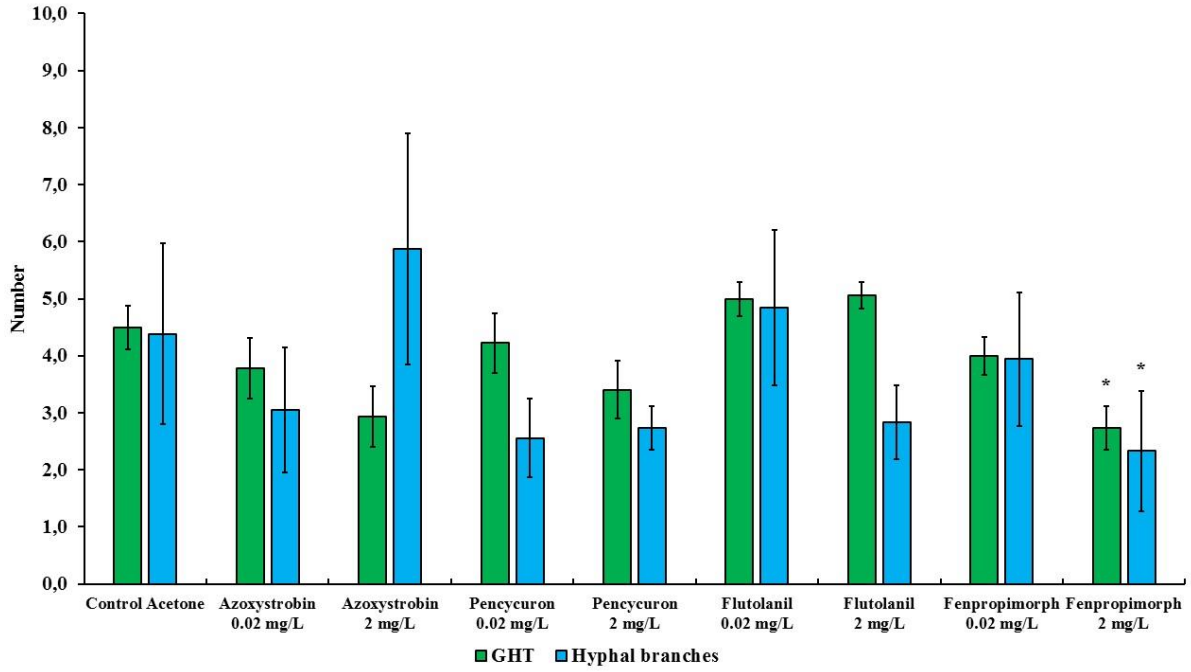

Fig. 24. Number of growing hyphal tips (GHT) and their hyphal branches produced in the hyphal healing mechanism (HHM) of *Rhizophagus irregularis* MUCL 41833 in absence (control acetone) or in presence of increasing concentrations of azoxystrobin, pencycuron, flutolanil and fenpropimorph (0.02 and 2 mg L^{-1}). Hyphal branches were numbered 48h after hyphal physical injury. Data are presented means ± standard error (two Petri plates per treatment with several injuries in each plate) and were analyzed by a Kruskal-Wallis test followed by a Dunn multiple comparison post-hoc test d to validate significant difference between fungicides treatments and their respective control acetone: *, ** and *** indicate P < 0.05; 0.01 and 0.001, respectively.

Discussion

Seed dressing or foliar application of chemical fungicides are considered essential agricultural practices to control fungal diseases in crops intended for market. They have different modes of action and can target a broad range or a specific group of fungi, but in numerous cases, they also affect non-target fungi. It is important to understand the effects of fungicides on these non-

target organisms, because some of them (e.g. AM fungi) play key roles in plant growth and health and thus help to optimize strategies of fungicide applications. Here, the effects of fungicides with different modes of action were investigated on the HHM, a strategy allowing fungi to preserve their mycelial networks integrity, of two AM fungi (*Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833) with contrasting life history strategies.

In the absence of fungicides, similar results to those obtained in the studies of de la Providencia et al. (2005) and de la Providencia et al. (2007) on *Glomeraceae* and *Gigasporaceae* grown *in vitro* were obtained, demonstrating that the approach considered in the present study was adequate for studying the HHM in presence of fungicides. These authors suggested that in *Gigasporaceae*, the HHM (i.e. GHTs contact and subsequent cytoplasmic/protoplasmic flow re-establishment) is the most probable way to maintain the viability of hyphae in adverse conditions. In contrast, in *Glomeraceae*, the HHM might increase the ability of the fungus to colonize the roots due to the proliferation of new branches at the apex of the injured hyphae as well as the reconnection of the affected area by networking several hyphae in a relatively small vicinity.

In the presence of fungicides, differences were noticed between active ingredients (a.i.) and concentrations. Azoxystrobin was detrimental to both fungi at the concentration of 2 mg L^{-1} but not at 0.02 mg L^{-1}. Pencycuron and flutolanil at both concentrations did not impact the HHM of the two fungi, while fenpropimorph had contrasting effects on both fungi. With *R. irregularis*, the HHM was stimulated at 0.02 mg L^{-1} and inhibited at 2 mg L^{-1} fenpropimorph, while no effect was observed at both concentrations in *Gigaspora* sp.

Azoxystrobin at 2 mg L^{-1} was the most threatening a.i. impacting all the HHM events in *Gigaspora* sp. and the contact and fusion of GHTs in *R. irregularis*, while at 0.02 mg L^{-1} no effects were noticed on any of the fungi. At the highest concentration of 2 mg L^{-1}, an evident retraction of cytoplasm in the opposite direction to the tips and subsequent formation of numerous walls inside the hyphae was observed when GHTs were not produced [70%] or did not enter into contact [85%] in *Gigaspora* sp. In *R. irregularis*, the GHTs [87.5%] were produced but only a few contacts [37.5%] and fusions [31.2%] were observed and the hyphae that did not form GHTs presented a main septum in the lesion area and did not produce numerous walls as in *Gigaspora* sp. These results complement the observations made by Buysens et al. (2015) on the effect of azoxystrobin on AM fungi. These authors demonstrated under similar *in vitro* culture conditions that azoxystrobin at 0.1 mg L^{-1} (as formulation Amistar) affected spore production and hyphal length of *R. irregularis* MUCL 41833 associated with potato, while root colonization was reduced at 1 (a.i. or formulation Amistar) and 10 mg L^{-1} a.i. Greenhouse studies further demonstrated that soil drench with azoxystrobin inhibited root colonization of *Glomeraceae* members in sugarcane (Vuyyuru et al. 2018). Moreover, root colonization and enzymatic activity of *Funneliformis coronatum* associated with maize plants was also inhibited, demonstrating the fungicidal activity of this strobilurin, probably on the respiratory electron transfer within mitochondria, where succinate dehydrogenase is part of complex II (Diedhiou et al. 2004). Interestingly, foliar application of azoxystrobin did not impact AM fungal root colonization (Diedhiou et al. 2004; Hernández-Dorrego and Mestre-Parés 2010; Campos et al. 2015). A similar trend was observed with kresoxim-methyl, another strobilurin fungicide (Diedhiou et al. 2004). Both strobilurin fungicides present low uptake into leaves and absence of phloem mobility (Bartlett et al. 2002) probably explaining the absence of effects on AM fungi in the roots. Interestingly, Buysens et al. (2015) evaluated that 0.75 mg L^{-1} azoxystrobin applied *in vitro* could be considered close to the recommended dosage of Amistar applied against *R. solani* in potato crop production (1500 g a.i. ha^{-1}) (Service Public

Fédéral 2015). Therefore, the concentration of 2 mg L^{-1} of a.i. used at the place of hyphal injury was probably above the recommended field dosage, while 0.02 mg L^{-1} was below. From these results, it is speculative to assert that field recommended dosage may impact the fungus significantly, because several aspects (e.g., biodegradation, soil type and soil particles size) might affect fungicide exposure to AM fungi (O'Connor et al. 2002; Rivera-Becerril et al. 2017). However, our results complement those obtained by Buysens et al. (2015), suggesting that the direct application in the culture medium of azoxystrobin at concentrations equal or above 0.1 mg L^{-1} (a.i. or formulated product) affect the development of AM fungi, therefore not excluding an impact on its development under field conditions following soil application.

The strong negative impact of azoxystrobin at 2 mg L^{-1} on the HHM (in particular contact and fusion of GHTs) of both AM fungi could possibly been attributed to the perturbation of apical vesicular bodies in the hyphal tip called Spitzenkörper. This structure consists in many small vesicles, ribosomes and cytoskeletal elements present in growing hyphal tips (Steinberg 2007) playing key roles in hyphal growth, orientation, branching and morphogenesis (Harris et al. 2005). This structure has not formally been described in AM fungi. Although, in *Gigasporaceae*, a cluster of spherical lipid bodies was observed behind the apex of growing germ tubes (Bentivenga et al. 2013) as well as 'spitzenkörper-like' structures at the hyphal apex of *Gigaspora margarita* (Ramos et al. 2008a, 2008b). In *Glomeraceae* their presence has never been proved but only suggested (de la Providencia et al. 2005). The perturbation of these structures by chemicals could explain the lack of recognition between GHTs in *R. irregularis* and *Gigaspora* sp. The effects of chemical molecules with fungicidal effects on the Spitzenkörper remains poorly investigated. Carbendazim, an inhibitor of ß-tubulin assembly in mitosis, was reported to inhibit completely the Spitzenkörper on living hyphal tip cells in *Fusarium acuminatum* (Ascomycota) (Howard and Aist 1977, 1980). Instead, in *Sclerotium rolfsii* (Basidiomycota), the Spitzenkörper position was displaced within the apical hyphal zone changing the growth direction when hyphae were treated with cyproconazole, an inhibitor of ergosterol synthesis, causing alteration of the microtubule cytoskeleton and abnormal wall deposition (Roberson et al. 1989). These studies demonstrate that chemicals may impact growth and orientation of hyphae, hence affecting other processes such as anastomosis between branches of intact hyphae or contact and fusion of GHTs in injured hyphae. Although this has to be demonstrated for those fungi and extended to AM fungi with molecules such as azoxystrobin.

Pencycuron at 0.02 and 2 mg L^{-1} did not impact the HHM of both AM fungal strains. Buysens et al. (2015) also demonstrated that pencycuron at 0.01 mg L^{-1} a.i. did not affect the ERM development or root colonization. To the contrary, at the concentration of 0.5 mg L^{-1} a.i. or formulation and 5 mg L^{-1} a.i., root colonization of *R. irregularis* was reduced. One, 10, 100 mg L^{-1} a.i. or formulation further impacted spore germination (Buysens et al. 2015). These authors calculated that a concentration of 0.25 mg L^{-1} used *in vitro* was equivalent to the recommended dose of Monceren applied in the field to control *R. solani* in potato crop production (500 g ha^{-1} a.i.). Our results suggested that the direct application of pencycuron at 0.02 and 2 mg L^{-1} had no noticeable effects on the HHM, while higher concentrations above the recommended dose calculated *in vitro* may affect other processes (e.g., spore germination, root colonization) of AM fungal life cycle. Pencycuron is the only fungicide of the phenylurea group, which also includes herbicides for weed control in agricultural and non-agricultural systems (Navarro et al. 2012) and is known to inhibit cell division of *Rhizoctonia* sp. The absence of effect of pencycuron on the HHM at concentrations up to 2 mg L^{-1} may suggest that the enzymes involved in cell division in AM fungi are less sensitive than those in *Rhizoctonia* sp. A study under greenhouse conditions nonetheless demonstrated that the cytokinin-like growth regulator

thidiazuron (phenylurea compounds) applied on leaves at 20 mg L^{-1} (91 µM) decreased root colonization of *R. intraradices* associated with the perennial grass *Miscanthus giganteus* (Schmidt et al. 2017), suggesting an indirect effect on AM colonization by a decrease in active cytokinines in the plant shoot. A modification in plant metabolism by the application of pencycuron on the leaves may thus impact AM fungal colonization more severely than a direct contact with the molecule.

Flutolanil at both concentrations did not affect the HHM of *Gigaspora* sp. And *R. irregularis*. This corroborates partially the study of Buysens et al. (2015). These authors noticed that flutolanil at 0.1 mg L^{-1} a.i. did not affect ERM development, while spore germination was reduced at 10 and 100 mg L^{-1} a.i. or formulation Monarch and root colonization decreased at 1 mg L^{-1} a.i. or formulation Monarch and 10 mg L^{-1} a.i. They suggested that flutolanil was more detrimental to the intraradical structures (hyphae and arbuscules) than ERM structures because it is an inhibitor of the succinate dehydrogenase and has a high systemic activity. Systemic fungicides are thought to be more detrimental to AM fungi because of their accumulation in or on the roots (Diedhiou et al. 2004; Burrows and Ahmed 2007; Jin et al. 2013). The absence of impact of flutolanil at 0.02 and 2 mg L^{-1} on the HHM of AM fungi suggest that the enzymatic metabolisms of the respiration pathway in AM fungi are probably less sensitive to flutolanil than those of Basidiomycota plant pathogens. In addition, 0.02 and 2 mg L^{-1} concentrations could be considered lower or higher respectively than the recommended dose reported by Buysens et al. (2015). These authors hypothesized a value of 0.09 mg L^{-1} *in vitro* considering the recommended dose of Monarch applied to control *R. solani* in potato crop production (184 g ha^{-1} a.i.). Our results thus suggest a tolerance of AM fungi to the direct application of flutolanil even at a concentration above the recommended dose estimated *in vitro*.

Fenpropimorph at the highest concentration impacted the HHM in *R. irregularis*, while the opposite was noticed at the lowest concentration. Curiously, no effect was observed on *Gigaspora* sp. It is not excluded that these differences may be related to the sterol composition of both fungi. Indeed, a variation in the composition and content of sterols has been observed in the spores of AM fungal species in the *Glomeraceae*, *Gigasporaceae* and *Acaulosporaceae* families (Grandmougin-Ferjani et al. 1999), not excluding the possibility of differences in the GHTs.

Sterols accumulation at the fungal growing tips support hyphal growth facilitating apical endocytosis and organizing the cytoskeleton (Steinberg 2007). Differences in sterol composition in GHTs may lead to differences in sensitivity to sterol biosynthesis inhibitors fungicides as fenpropimorph. In *R. irregularis*, GHT production was not affected by the high concentration of fenpropimorph, but reduced contacts and thus fusions were observed, suggesting perturbation of the GHT recognition as with azoxystrobin. A common attraction (positive tropism) between GHTs was observed with *Gigasporaceae* members and suggested in *Glomeraceae* (de la Providencia et al. 2005). These authors hypothesized that the growth of both GHTs towards each other was due to elicitation of diffusible substance (e.g., 'hormones' or enzymes and proteins delivered from Spitzenkörper) and that this mechanism was led by one GHT in a sequence of a signaling response.

The stimulatory effect observed on HHM of *R. irregularis* at low concentration (0.02 mg L^{-1}) and opposite observed at high concentration (2 mg L^{-1}) of fenpropimorph could be related to a phenomenon named hormesis. The hormetic effect is a biphasic dose response characterized by temporal stimulation at low doses and inhibition at high doses of a stressor agent, suggesting an adaptive response that can be either directly induced or be the result of overcompensation

after the alteration of homeostasis in a biological process (Calabrese and Baldwin 2002). The hormetic effect of fungicides on mycelial growth and virulence have been previously reported in Oomycota, Ascomycota and Basidiomycota plant pathogens (Flores and Garzon 2013; Lu et al. 2018; Zhang et al. 2019). To the best of our knowledge, hormesis has never been addressed in Glomeromycota and deserve attention owing to the importance of these organisms for crops (Hage-Ahmed et al. 2019).

A strong impact of fenpropimorph at concentrations equal or above 0.2 mg L^{-1} under *in vitro* culture conditions was observed by Campagnac et al. (2008) and Zocco et al. (2008) on ERM development of *R. irregularis* (hyphal growth, spore production and mycelium architecture). This impact resulted in a decreased capacity of the fungus to transport P to the host plant and a drastic decrease of alkaline phosphatase and succinate dehydrogenase activities measured in the ERM (Zocco et al. 2011). With the same AM fungus, an induction of oxidative stress was observed in presence of fenpropimorph at 10 mg L^{-1}, suggesting the perturbation of other biological processes such as response to biotic and abiotic environmental stimuli (González-Guerrero et al. 2010).

In conclusion, this work reports for the first time the effects of fungicides with different modes of action on the HHM in two phylogenically-distant AM fungi (*R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331).

Azoxystrobin (a broad-spectrum fungicide) was the most detrimental on both fungi at concentration slightly above the recommended field dosage. Conversely, the contact fungicide pencycuron (*Rhizoctonia*-specific) and flutolanil (Basidiomycota-specific) did not impact any of the two AM fungi, while fenpropimorph only impacted *R. irregularis* (stimulating at low and inhibiting at high concentrations). The mechanisms behind the observed results remain to be elucidated, but perturbation in the still-to-be firmly demonstrated spitzenkörper or sterols content as well as a process of hormesis are avenues to be explored. Our results on HHM/anastomosis process complement previous studies on the impact of pesticides on root colonization or spore germination/production and broadens our knowledge on the impact of fungicides on AM fungi and opens new avenues for the rationale management of chemical inputs to control pathogenic fungi while limiting their impact on AM fungi. Other research lines must be explored about the resilience capacity of AM fungi with different LHS after soil disruption in field or in zero tillage systems, but with a high dependence on pesticide use. It also increases our knowledge of the different strategies for AM fungal colony to survive under adverse conditions in agricultural soils.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Victor Hugo Rodriguez-Morelos: Data collection, analysis, and interpretation, drafting the work, commentaries corrections, final approval, and agreement with all aspects of the work. Maryline Calonne-Salmon: Data collection, analysis and interpretation, draft work commentaries and corrections, final approval, and agreement with all aspects of the work. Vincent Bremhorst: Analysis and interpretation of the data, draft correction and final approval and agreement with all aspects of the work MG-R: Contribution to the development of the

experiment, draft correction and final approval and agreement with all aspects of the work. Stéphane Declerck: Substantial contributions to the conception and design of the experiments, interpretation of the data, draft corrections final approval and agreement with all aspects of the work.

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Preface

Herbicides are major compounds used to control weeds in agroecosystems. Nowadays glyphosate has become the most widely applied pesticide in the world. However, its application can also impact non-target beneficial soil microorganisms such as AM fungi either directly by contact with the fungi in soil or indirectly, by killing their host plant. Nowadays scientific knowledge on this interaction remains not fully understood and their effect on the process of anastomosis process is totally unknown.

In chapter I, fungicides active ingredients (a.i.) with different modes of action were tested under *in vitro* conditions on the HHM of *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833. The type of fungicide and concentration (0.02 and 2 mg L^{-1}) impacted differentially both strains. Azoxystrobin was the most detrimental for both AM fungi at 2 mg L^{-1}. Curiously, fenpropimorph only impacted *R. irregularis* (stimulating at low and inhibiting at high concentration). In contrast, flutolanil and pencycuron did not impact any of the two AM fungi.

In **chapter II**, the objective was to investigate under similar *in vitro* culture conditions as in chapter I, the impact of glyphosate at increasing concentrations (i.e., 0.02, 0.2 and 2 mg L^{-1} a.i.) on the HHM in *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833. As in chapter I, four events in the process of HHM (septum formation, emission, contact and fusion of growing hyphal tips) were monitored at regular intervals under a dissecting bright-field light microscope.

Chapter II

Glyphosate differently affects the hyphal healing mechanism in *Gigaspora* sp. and *R. irregularis*

Short note in preparation for Mycorrhiza

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Abstract

Herbicides are widely used in conventional agriculture to control weeds but may also affect non-target microorganisms such as arbuscular mycorrhizal (AM) fungi. These symbionts develop extensive mycelial networks within the soil via mechanisms such as anastomosis which indiscriminately concern intact and damaged hyphae, the latter being referred to as the hyphal healing mechanism (HHM). The HHM differs between AM fungal families. However, the effects of herbicides on this mechanism in totally unknown. Here, the effect of glyphosate at increasing concentrations (0.02, 0.2, 2 mg L^{-1}) was tested *in vitro* on the HHM in *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833. Glyphosate at 2 mg L^{-1}, stimulated the emission and contact of growing hyphal tips (GHT) in *R. irregularis*, while no impact was noticed in HHM event in *Gigaspora* sp. These results support the idea that AM fungi with different LHS express variable sensitivity to glyphosate during hyphal healing. The specific biochemical mechanisms involved behind the observed results remain to be elucidated.

Keywords

Arbuscular mycorrhizal fungi, Extraradical mycelium, Pesticides, Herbicides, Anastomosis

INTRODUCTION

The extraradical mycelium (ERM) of arbuscular mycorrhizal (AM) fungi is fundamental in plant growth and maintenance of soil biological fertility (Avio et al. 2006; Pepe et al. 2018; de Novais et al. 2019a). Due to its intrinsic belowground nature, the ERM is continuously reconfigured in response to niche-specific nutritional and environmental changes and to physical damage, through a combination of hyphal extension, branching and interconnection by means of hyphal fusion. This last process, also termed anastomosis (i.e., the *fusion between branches of the same or different hyphae to produce a mycelial network that involves degradation of the cell wall, followed by plasma membrane fusion*" (Glass and Fleissner 2006)), is crucial for the growth, maintenance/survival, and interconnectedness of mycorrhizal mycelium (de la Providencia et al. 2005). The process indistinctly concerns intact and damaged hyphae, the latter being referred to as the hyphal healing mechanism (HHM). The HHM has been described in detail for AM fungal species belonging to the *Glomeraceae* and *Gigasporaceae* families. Four successive events could be separated: (1) septum formation, (2) emission, (3) contact and (4) fusion of growing hyphal tips (GHTs) (de la Providencia et al. 2005). Importantly, the HHM differs between both families. In *Glomeraceae* this mechanism is oriented towards the reconnection of the affected area by linking several hyphae in relatively small vicinity or by recolonization of roots and substrate, while in *Gigasporaceae* it is oriented to the re-establishment of hyphal integrity (de la Providencia et al. 2007). The contrasting responses of both fungal families suggest that AM fungi have developed different strategies to survive and colonize plants under adverse conditions. Curiously, *Gigasporacea* tend to disappear in intensive cultivated soils, while *Glomeraceae* seems more resistant to agricultural practices (Antunes et al. 2006; Soka and Ritchie 2018). Studies suggested that the viability of AM fungal structures (e.g., spores and ERM) could be affected when exposed to active substances of pesticides (Rivera-Becerril et al. 2017; de Novais et al. 2019a). Nowadays, glyphosate is the most widely applied herbicide worldwide used to control weeds, because of the increase of genetically engineered herbicide-tolerant crops (Benbrook 2016). This herbicide is also a critical tool in no-till cropping systems (Schlatter et al. 2017). Glyphosate disrupts the 5-enolpyruvylshikimate-3-phosphate synthasephosphate synthase, a key enzyme of the shikimate-pathway in plant photosynthesis (Duke and Powles 2008).

The effects of this herbicide on AM fungi are mixed. Several studies have reported the absence of detrimental effects under greenhouse or field conditions (Malty et al. 2006; Savin et al. 2009; Powell et al. 2009; Baumgartner et al. 2010; Brito et al. 2013; Gornish et al. 2020, Sesin et al. 2021). In contrast, a decrease in root colonization and spore viability and production has been noticed in other studies (Baumgartner et al. 2005; Druille et al. 2013a, 2013b, Carvalho et al. 2014; Zaller et al. 2014; Druille et al. 2015; Druille et al. 2017; Helander et al. 2018; Nivelle et al. 2018; Zaller et al. 2018; Gu et al. 2019; Cruz et al. 2020; Wilkes et al. 2020; Malfatti et al. 2021). These contrasting results have been attributed to the type of application, plant host and the interaction with other agricultural practices (e.g., plowing or fertilization) (Ronco et al. 2006; Savin et al. 2009; Pasaribu et al. 2011; Sheng et al. 2012; Maltz et al. 2016; Wilkes et al., 2020). Additionally, evidence supports that glyphosate application impacts AM fungi directly, through contact with spores and ERM in soil, and indirectly, by changes generated in the host plant (Druille et al. 2013a, 2013b). However, the direct effect of glyphosate on the HHM is totally unknow. Therefore, the objective of this study was to investigate under *in vitro* culture conditions the impact of different concentrations of glyphosate on the HHM of AM fungi with different life history strategies (*Gigaspora* sp. and *R. irregularis*).

Materials and methods

Biological material

The AM fungi *Gigaspora* sp. (Gerdemann and Trappe) MUCL 52331 and *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] MUCL 41833 were supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). Both strains were maintained in association with Ri T-DNA transformed chicory (*Cichorium intybus* L.) and carrot (*Daucus carota* L.) roots respectively on 135 mm diam. Petri plates containing 100 ml Modified Strullu–Romand (MSR) medium (Declerck et al. 1998).

Glyphosate medium preparation

The glyphosate active ingredient (a.i.) was supplied by Sigma-Aldrich, Inc. [Buchs Switzerland]. The herbicide was dissolved in a solution of water (5 ml L^{-1} of MSR medium) and added to a bottle containing 50 ml sterilized (121°C for 15 min) MSR medium. The a.i. was added at a concentration of 0.02, 0.2, and 2 mg L^{-1} MSR medium (Zocco et al. 2008). The bottles contained a stirring magnet to avoid solidification and maintain a homogeneous concentration of the a.i. One bottle containing MSR medium without glyphosate was used as control treatment.

Impact of glyphosate on the hyphal healing mechanism

Monoxenic cultures of both AM strains were achieved following the method described by Rodriguez-Morelos et al. (2021). Briefly, after 12 and 6 weeks of growth for *Gigaspora* sp. and *R. irregularis*, respectively, Petri plates showing homogeneous ERM development were selected. Between 5 and 10 hyphae growing on the surface of the MSR medium in the thinner part of the Petri plates and showing dense cytoplasmic/protoplasmic flow under a dissecting bright-field light microscope (Olympus SZ2-CTV, Japan) at 40 x magnification were selected in each Petri plate. The selected hyphae were chosen at a reasonable distance from each other to avoid as much as possible interferences (i.e. hyphae contact from different cuts). Under horizontal laminar hood, hyphae showing an active cytoplasmic/protoplasmic flow were cut with a sterilized scalpel under the dissecting bright-field light microscope at 6.7 x to 40 x magnification. Immediately after cutting, 20 µl of MSR medium without herbicide ($MSR^{control}$) or containing one of the three concentrations of glyphosate was added at the place of injury. The HHM was analyzed in injured hyphae belonging to two AM fungal cultures (i.e. two Petri plates) per strain (*Gigaspora* sp. and *R. irregularis*) and for the control treatment ($MSR^{control}$) and glyphosate concentration (0.02, 0.2 and 2 mg L^{-1}). Between 5 and 10 hyphae were considered per Petri plate. The cut hyphae were observed cautiously at regular intervals under the dissecting bright-field light microscope at 100 x or 200 x magnification, first frequently during the first 60 min, then each hour until 16 h and finally after 24, 30, 36 and 48 h.

The hyphae were visualized carefully under a dissecting bright-field light microscope (Olympus BH2–RFCA, Japan) at 40 x or 100 x magnification. Following hyphae cutting, four events in the HHM (de la Providencia et al. 2005) were monitored: (1) septum formation (result not shown); (2) emission of GHT through the septum or behind it and production of new branches on the GHTs; (3) elongation, orientation, and contact of the GHTs and (4) fusion and re-establishment of cytoplasmic/protoplasmic flow. The percentages of observed events within each treatment are described in Table 1.

Statistical analysis

A Cox proportional hazard model for right and interval censored (time-to-event) data was performed to evaluate the impact of glyphosate on HHM events, each hyphal injury from all treatments, was considered. Specifically, the *MIICD.coxph* function of the Multiple Imputation for Interval Censored Data R package was used (Delord 2015). The non-observed events after 48 h were considered as right censored. The hazard ratio's (hr) reported in Table 2 and defined as exp (β), where the β's are the coefficient estimates obtained from the fit of the Cox model, are interpreted as follows: an hazard ratio greater (respectively smaller) than one means that the event is more (respectively less) likely (i.e. the event occurs more (respectively less) often or is sooner (respectively later) observed) within the given treatment group compared to the control treatment. All data were analyzed with R software version 4.1.1. The statistical significance was established at a 95% confidence level (i.e. when the *P*-value is less or equal to 0.05).

Results

Hyphal healing mechanism in *Rhizophagus irregularis* and *Gigaspora* sp

In the absence of glyphosate, both AM fungi showed loss of cytoplasmic/protoplasmic content at both ends of the cut hyphae. Then a septum was formed and a darkening inside the hyphae a few microns from the cut ends was noticed within the first 180 minutes. Differences were observed between both AM fungi, especially in the number of GHT produced. In *Gigaspora* sp., a low number of GHT (with few hyphal branches) was noticed emerging behind both extremities of the injured hyphae. In most of the cases the GHTs grew towards each other, reconnected and reestablished cytoplasmic/protoplasmic flow. In contrast, *R. irregularis* produced a higher number of GHTs at both extremities growing throughout the septum and producing several hyphal branches. Hyphal re-growth appeared disorganized with a lower percentage of reconnections as compared with *Gigaspora* sp.

Hyphal healing mechanism in *Rhizophagus irregularis* and *Gigaspora* sp. in presence of glyphosate

The impact of glyphosate at 0.02, 0.2 and 2 mg L^{-1} was assessed on the HHM in *R. irregularis* and *Gigaspora* sp. (**Tables 9 and 10**). The results obtained from the Cox model on HHM of *R. irregularis* revealed that the glyphosate at 2 mg L^{-1} treatment had a significant impact on GHTs emission and contact events as the hazard ratio's were significantly above 1 (hr = 2.689, P = 0.008 and hr = 3.510, P = 0.001, respectively) (**Table 10**). These results mean that both events were observed more quickly within the glyphosate at 2 mg L^{-1} treatment compared to the MSR control one. However, at the end, the percentages of GHTs emission and contact were almost equal to $MSR^{control}$ (**Table 9**). In contrast, no significant effect of the glyphosate at 2 mg L^{-1} on GHT fusion was found compared to the control treatment. The GHTs emission, contact and fusion events were not significantly affected by the lower concentrations of glyphosate (i.e., 0.02 mg L^{-1} and 0.2 mg L^{-1}) compared to the control treatment (**Table 10**). Whatever the concentration of glyphosate, the emission, contact and fusion of GHTs in *Gigaspora* sp. were not affected compared to the control treatment according to the Cox Model (**Table 10**). Indeed, although a decreased trend in the percentage of observed GHTs emission, contact and fusion events was noted in presence of glyphosate concentrations, no significant difference was reported by our analysis (**Tables 9** and **10**).

Table 9. Data percentages calculated from the total of observations in each treatment of hyphal branches production, growing hyphal tips (GHT) emission, contact and fusion of the hyphal healing mechanism (HHM) of *Gigaspora sp.* MUCL 52331 and *Rhizophagus irregularis* MUCL 41833 in absence (control acetone) or in presence of increasing concentrations of glyphosate (0.02, 0.2 and 2 mg L^{-1}).

	***Gigaspora* sp. MUCL 52331**				***Rhizophagus irregularis* MUCL 41833**			
Events	**Control**	**0.02 mg L^{-1}**	**0.2 mg L^{-1}**	**2 mg L^{-1}**	**Control**	**0.02 mg L^{-1}**	**0.2 mg L^{-1}**	**2 mg L^{-1}**
GHT emission (%)	100	85.7	94.4	92.3	100	88.8	93.7	93.7
GHT contact (%)	100	78.5	77.7	76.9	88.2	77.7	81.2	87.5
GHT fusion (%)	93.3	71.4	77.7	76.9	76.5	72.2	81.2	75
Number of injured hyphae (n)	15	14	18	14	17	18	16	16

Table 10. Results of Cox proportional hazards regression analysis on growing hyphal tips (GHT) emission, contact and fusion events of the hyphal healing mechanism (HHM) for *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833 in presence of increasing concentrations of glyphosate (0.02, 0.2 and 2 mg L^{-1}).

		***Gigaspora* sp. MUCL 52331**			***Rhizophagus irregularis* MUCL 41833**		
Events	**Glyphosate concentration (mg L^{-1})**	**β**	**Hazard ratio**	**P - Value**	**β**	**Hazard ratio**	**P - Value**
GHT emission	0.02	0.109	1.115	0.781	0.469	1.599	0.180
	0.2	-0.487	0.614	0.179	0.392	1.479	0.271
	2	-0.022	0.978	0.955	0.989	2.689	**0.008**
GHT contact	0.02	-0.256	0.774	0.530	0.581	1.787	0.121
	0.2	-0.470	0.625	0.222	0.577	1.781	0.134
	2	-0.197	0.821	0.637	1.255	3.510	**0.001**
GHT fusion	0.02	-0.276	0.758	0.505	0.612	0.403	0.129
	0.2	-0.683	0.505	0.075	-0.454	0.396	0.251
	2	-0.371	0.690	0.373	-0.038	0.402	0.924

Values were considered significant at $P < 0.05$ compared to the control treatment.
β = Regression coefficient.
Hazard ratio = Exp (β).

Discussion

Glyphosate is by far the most widely used herbicide in agriculture to control weeds. Its high solubility in water and high mobility in soils allows its rapid leaching into the soil potentially affecting groundwater but also the microbial flora. To date, scientific studies on the impact of glyphosate on soil organisms (e.g., rhizobacteria, earthworms, endophytic fungi) have provided contrasting results (Brito et al. 2013, Druille et al. 2013a, 2013b; Druille et al. 2015; Helander et al. 2018; Wilkes et al. 2021; Vázquez et al. 2021). This is true as well for AM fungi. Reduced spore viability and root colonization has been observed in some studies (Malty et al. 2006; Zaller et al. 2014; Druille et al. 2015), while other reported null impact (Powell et al. 2009; Reis et al. 2014; Malfatti et al. 2021). Recently, greenhouse studies reported a strong impact of herbicides (e.g., dicamba, glufosinate-ammonium and pendimethalin) on ERM viability and anastomosis in *Funneliformis mosseae* (de Novais et al. 2019a, 2019b). However, none focused on glyphosate and its potential impacts on mechanisms such as anastomoses and HHM.

Here we observed that glyphosate stimulated the emission and contact of GHTs in *R. irregularis* MUCL 41833 at the concentration of 2 mg L^{-1}, but did not affect the fusion of GHTs, while at lower concentrations (0.02 and 0.2 mg L^{-1}) no impact was observed on emission, contact and fusion of GHTs. Conversely, whatever the concentration of glyphosate tested, no impact was noticed on emission, contact and fusion of GHTs in *Gigaspora* sp. MUCL 52331. These results suggest a differential tolerance or response mechanism between a *Glomeraceae* and

Gigasporaceae species to glyphosate exposure. A similar observation was made by Rodriguez-Morelos et al. (2021) with fenpropimorph, a systemic sterol biosynthesis inhibitor fungicide. These authors noticed, with the same two AM fungi and *in vitro* growth conditions, an absence of effect on HHM in *Gigaspora* sp. MUCL 52331, while with *R. irregularis* MUCL 41833, a stimulation was noticed at low concentration (0.02 mg L^{-1}) and inhibition at high concentration (2 mg L^{-1}) of fenpropimorph. They suggested that these differences may be related to the sterol composition of both AM fungi. Interestingly, Wilkes et al. (2020) demonstrated that glyphosate soil application decreased ergosterol levels used as a proxy for fungal biomass and suggested a negative impact on AM fungal growth.

Our results suggest an absence of inhibitory effect of glyphosate on the HHM in both AM fungi. We hypothesize that the signals that contribute to the emission and then contact and fusion of GHTs, are not disturbed or masked by the presence of the herbicide a.i. However, it cannot be ruled out that the a.i. of glyphosate has diffused into the gelled medium, therefore reducing the concentration of the active molecule in the close vicinity of the cut hyphae. In parallel, the release of diffusible substances by GHTs during the process of healing is a continuous process with, on the contrary, an increasing concentration as the GHTs approach each other. Therefore, this may explain why glyphosate did not inhibit the HHM in both AM fungi.

It is often reported that the fusion of hyphae is linked to the presence of apical vesicular clusters (AVC – also named spitzenkörper). Although we did not explore the possible presence of these AVCs in our study, it seems probable that glyphosate did not impact their occurrence and function in the hyphal tips in contrast to fungicides such as azoxystobin impacting contact and fusion of GHTs at the concentration of 2 mg L^{-1}, probably because of the perturbation of AVCs in the hyphal tips (Rodriguez-Morelos et al. 2021).

Curiously, the a.i. of glyphosate at the highest concentration stimulated the emission and contact of GHTs in *R. irregularis* MUCL 41833, while it has no impact on *Gigaspora* sp. MUCL 52331. It is not excluded that this may be related to a stress reaction characterized by the faster production of GHTs. Obvisouly, under no-stress conditions, *R. irregularis* differs from *Gigaspora* by a much higher number of GHTs emitted (de la Providencia et al. 2005). This is related to their strategy to heal oriented towards the production of numerous GHTs increasing the possibilities of contact of the affected area by linking several hyphae in relatively small vicinity or by recolonization of roots and substrate between injury hyphae. Instead, *Gigaspora* members produce a much more limited number of GHTs at the extremities of the hyphae oriented towards the re-establishment of hyphal integrity as suggested de la Providencia et al. (2007). The emission of GHTs in *R. irregularis* MUCL 41833 may be accelerated in presence of glyphosate a strategy in cell fungal membrane to avoid first cytoplasma loss and then herbicide contamination allowing the contact and healing of ERM in this *Glomeraceae* member.

In conclusion, this work reports for the first time a differential tolerance of two phylogenically-distant AM fungal strains (*R. irregularis* and *Gigaspora* sp.) to glyphosate exposure on the HHM. Here, the concentrations used were below and above the recommended field dosage of glyphosate (~ 0.8 mg L^{-1} *in vitro*) calculated considering conventional application in soybean crops by Moorman (1986). From the present study, it is hypothesized that glyphosate above recommended dosage present low or no inhibitory impact on the HHM of AM fungi in the field, because many factors may affect the concentration of the herbicide close to the fungus (e.g., dilution effect in the soil volume, physicochemical characteristics of the soil, degradation of the

a.i. by microorganisms etc.). Further studies are, anyway, necessary to unravel the mechanisms involved in the stimulation of GHTs emission and contact in *R. irregularis* and its absence in *Gigaspora*.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Victor Hugo Rodriguez-Morelos: Data collection, analysis, and interpretation, drafting the work, commentaries corrections, final approval, and agreement with all aspects of the work. Maryline Calonne-Salmon: Data collection, analysis and interpretation, draft work commentaries and corrections, final approval, and agreement with all aspects of the work. Stéphane Declerck: Substantial contributions to the conception and design of the experiments, interpretation of the data, draft corrections final approval and agreement with all aspects of the work.

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Preface

Arbuscular mycorrhizal (AM) fungi belonging to the *Glomeraceae* family, such as *Rhizophagus irregularis*, are widely distributed in croplands, developing large mycelial networks, via anastomosis, to explore and exploit their environment. However, the impact of fungicides on anastomosis formation of AM fungi has been scarcely studied.

In chapter I and II, the impact of fungicides (azoxystrobin, flutolanil pencycuron and fenpropimorph) extensively applied in cropping systems, and glyphosate, the herbicide most widely used around the world, were evaluated under *in vitro* culture conditions on the hyphal healing mechanism (HHM). In both chapters we focused on the HHM following physical damage on the extraradical mycelium (ERM) of *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833, two important AM fungi having contrasting life history strategies.

However, it is unknown whether those fungicides have an impact on the capacity of AM fungi To form anastomosis within and between mycelial networks in absence of physical injury. Therefore, the objective of **Chapter 3** was to investigate under *in vitro* culture conditions the impact of azoxystrobin, flutolanil, pencycuron and fenpropimorph at the concentrations used in chapter I on the capacity of *R. irregularis* MUCL 41833 to form anastomoses within and between mycelial networks in absence of physical damage. A tri-compartmented culture system was developed based on the system described by Voets et al. (2006), providing a nondestructive, three-dimensional visualization of the development pattern of the ERM of *R. irregularis* MUCL 41833. Then, the impact of fungicides on anastomosis within and between mycorrhizal networks, spore production and growth, were evaluated.

Chapter III

Anastomosis within and between networks of *Rhizophagus irregularis* is differentially influenced by fungicides

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Abstract

Arbuscular mycorrhizal (AM) fungi play key roles in soil fertility of agroecosystems. They develop dense extraradical mycelial (ERM) networks via mechanisms such as hyphal anastomosis. These connections between hyphae can be affected by agricultural practices such as the use of fungicides, but how these compounds affect anastomosis formation within and more importantly between networks of the same AM fungal strain remains poorly unexplored. Here, the impact of azoxystrobin, pencycuron, flutolanil, and fenpropimorph at 0.02 and 2 mg L^{-1} were tested *in vitro* on the anastomosis formation within and between networks of *Rhizophagus irregularis* MUCL 41833. Azoxystrobin and fenpropimorph had a particularly detrimental impact, at the highest concentration (2 mg L^{-1}), on the number of anastomoses within and between networks, and for fenpropimorph in particular at both concentrations (0.02 and 2 mg L^{-1}) on the number of anastomoses per length of hyphae. Curiously fenpropimorph at 0.02 mg L^{-1} significantly stimulated spore production, while with azoxystrobin, the reverse was observed at 2 mg L^{-1}. The two other fungicides, pencycuron and flutolanil, had no detrimental effects on spore production or anastomosis formation within and between networks. These results suggest that fungicides with different modes of action and concentrations differentially affect anastomosis possibly by altering the hyphal tips of AM fungi and may thus affect the capacity of AM fungi to develop large hyphal networks exploring and exploiting the soil at the service of plants.

Keywords

Arbuscular mycorrhizal fungi · Mycelial networks · Fungicide · Anastomosis

Introduction

Rhizophagus irregularis forms large mycelial networks via anastomosis between different branches of the same or different hyphae (de la Providencia et al. 2005; Kirk et al. 2008), ultimately connecting plants (Bücking et al. 2016; Wipf et al. 2019). The mechanism of anastomosis has essential functions in fungal mycelium such as heterokaryon formation, intra-hyphal communication, and homeostasis maintenance (Glass et al. 2000). In arbuscular mycorrhizal (AM) fungi, it allows mycelial growth and physical exploration of the environment forming a three-dimensional network with protoplasmic/cytoplasmic flow, genetic material exchange, and exchange of mineral nutrients (Giovannetti et al. 2001, 2015; Mikkelsen et al. 2008; Croll et al. 2009; Daubois et al. 2016; de Novais et al. 2017; Sbrana et al. 2018). This capacity to form anastomoses within and between mycelial networks represents an important mechanism evolved by AM fungi to fulfill their functions in nutrients uptake and transport to the plant. For instance, Avio et al. (2006) noticeda positive correlation between anastomosis frequency in *Glomeraceae* members and growth and P nutrition of *Medicago sativa*.

Agricultural practices such as the application of pesticides can affect spore germination and production, and viability of extraradical mycelium (ERM) (Zocco et al. 2008; Campagnac et al. 2009; Calonne et al. 2012) as well as diversity of AM fungi (Ren et al. 2021; Edlinger et al. 2022). Only a few studies (de Novais et al. 2019a; Rodriguez-Morelos et al. 2021), however, have reported the effects of fungicides on anastomosis formation within a mycelial network and no study, to the best of our knowledge, has reported the effects of fungicides on the capacity of AM fungi to form anastomoses between networks of the same isolate. The objective of this study was to investigate under *in vitro* culture conditions the impact of four fungicides (azoxystrobin, pencycuron, flutolanil, and fenpropimorph) with contrasting modes of action on the capacity of *R. irregularis* MUCL 41833 to form anastomoses within and between mycelial networks. *Rhizophagus irregularis* MUCL 41833 was selected because it belongs to a family, the Glomeraceae, (i) widely distributed in agricultural soils (Séry et al. 2018; Guzman et al. 2021) thus susceptible to grow in the presence of fungicides, and (ii) able to form anastomoses within (de la Providencia et al. 2007; Purin and Morton 2011; Pepe et al. 2018) and between (Voets et al. 2006; de la Providencia et al. 2013) mycelial networks.

Materials and methods

Biological material

Rhizophagus irregularis (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as "irregulare" MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO—http://www.mycorrhiza.be/ginco-bel) and maintained in association with Ri T-DNA transformed carrot (*Daucus carota* L.) roots on 90-mm diameter Petri plates containing 25 mL of Modified Strullu-Romand (MSR) medium (Declerck et al. 1998).

Fungicide medium preparation

Four fungicides were supplied by Sigma-Aldrich, Inc. (Darmstadt, Germany) as active ingredients: three systemic fungicides, azoxystrobin and flutolanil which disrupt respiration, and

fenpropimorph which inhibits sterol biosynthesis in membranes, and one contact fungicide, pencycuron, which affects the cytoskeleton and motor proteins (Fungicide Resistance Action Committee 2022). The active ingredients were tested at two concentrations (i.e., 0.02 mg L^{-1}, a low concentration which poorly affected the development of *R. irregularis*, and 2 mg L^{-1}, a high concentration which strongly affected the development of *R. irregularis* (Campagnac et al. 2008, 2009; Zocco et al. 2011)) in the MSR medium, following dissolution in acetone at a concentration of 5 mL^{-1} of MSR medium. Eight fungicide treatments were considered: azoxystrobin (0.02 and 2 mg L^{-1}), flutolanil (0.02 and 2 mg L^{-1}), pencycuron (0.02 and 2 mg L^{-1}), and fenpropimorph (0.02 and 2 mg L L^{-1}). Two control treatments were also included in the design: MSR medium without fungicides but supplemented with the same concentration of acetone ($MSR^{acetone}$) and MSR medium alone ($MSR^{control}$).

Experiment design

Impact of fungicides on the extraradical mycelium (ERM) networks of *R. irregularis*

Thirty millilitres of MSR medium was added to each LC,whereas the CC remained empty. One 1.5 × 1.5 cm sliced piece of gel containing a mycorrhizal root with ERM and approximately 250 spores from a 6-week-old root organ culture (ROC) of carrot was placed on the surface of the MSRmedium (Cranenbrouck et al. 2005) in both LCs (**Figure 26**). The culture plates were incubated in an inverted position at 27 °C in a growth chamber under dark conditions. During the incubation period, the root and mycelium growth was monitored every 48 h. The roots that crossed the partition walls separating the LCs from the CC were trimmed. After 4 to 6 weeks, the two LCs were covered with a dense ERM. The culture plates were selected for their homogeneous development ofmycelial network developing in each LC. At that time, the CC was filled with 30 mL of MSR medium without fungicide ($MSR^{control}$) or ($MSR^{acetone}$) or with each of the fungi- cide treatments (see above), to reach the top of the plastic walls separating the LCs with the CC. After a period of 15 days, the CC was covered with the ERM throughout the depth of the medium (**Figure 26**).

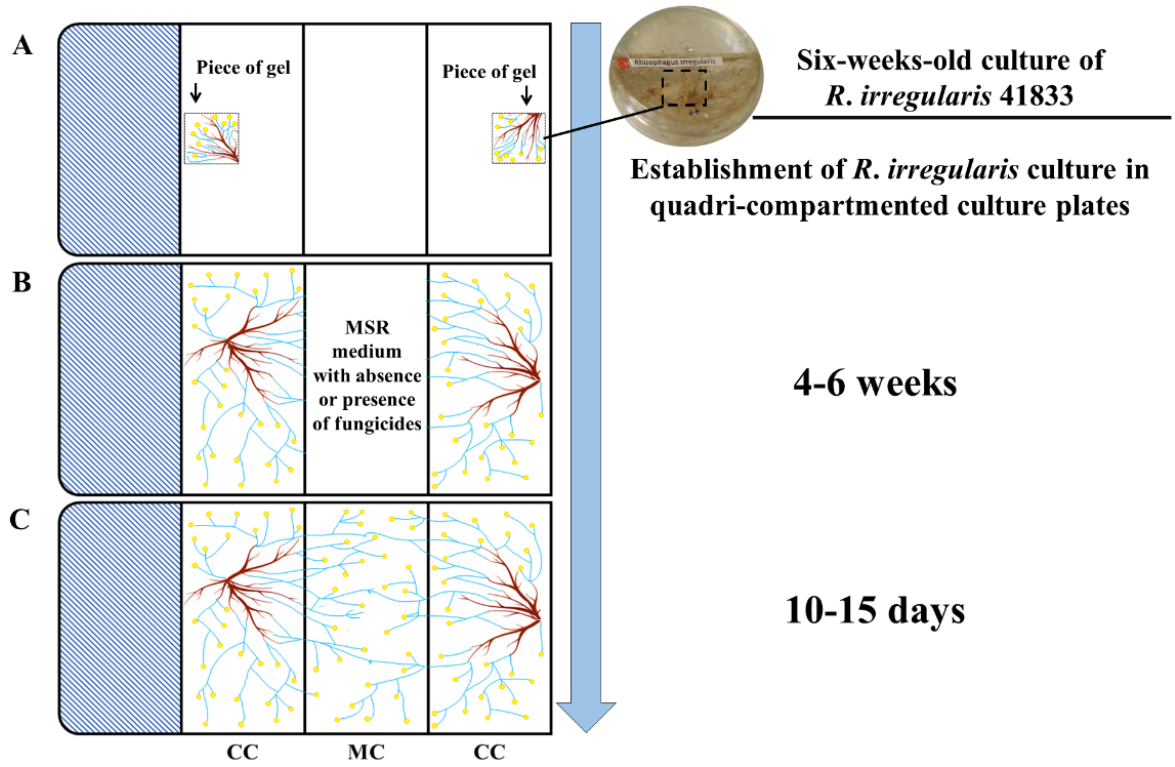

Fig. 26. Experimental process to assess the anastomosis formation within a same network or between two networks and ERM growth parameters of *R. irregularis* MUCL 41833 in absence ($MSR^{control}$ and $MSR^{acetone}$) or presence of increasing concentrations of fungicides (0.02 and 2 mg L^{-1}).

Different parameters were recorded in the CC: (1) the number of newly produced spores, (2) the total hyphal length, and (3) the number of anastomoses within the same network (i.e., originating from the same LC) and between networks (i.e., originating from the two intersecting mycelial networks developing from each LC).

In the medium, all anastomoses were numbered, although it was not always possible to verify possible incompatible fusions and non-active anastomoses. The anastomosis within and between networks of *R. irregularis* was carefully recorded following hyphae origin and according to the detailed description of Voets et al (2005). The total hyphal length was measured following the method detailed by Tennant (1975) and Declerck et al. (1998). Briefly, gridlines composed of squares of 1 cm^2 was placed on the bottom of the plates and intersections of hyphae with the gridlines were counted. The length of the mycelium was estimated according to the formula: length (cm) = (number of intersections*area of the compartment (cm^2)) / (2*length of lines of the grid (cm)). To determine the presence and type of anastomosis, the culture plates were observed under a dissecting microscope Olympus SZ2-ILST, Japan (6.7 to × 40 magnification) and a compound Olympus BH2-RFCA, Japan, with bright-field light illumination (× 40 or × 100 magnification).

The experiment was repeated twice with a one-month interval between both blocks (first and second blocks had three or two replicates, respectively). A total of five replicates were considered for each treatment that consisted of one culture plate with two CC and one MC where the ERM was allowed to develop and contact each other.

Statistical analysis

Levene and Shapiro–Wilk tests were used to confirm homogeneity of variance and normality in distributions of response variables, respectively. Both control treatments (i.e., $MSR^{acetone}$ and $MSR^{control}$) were compared using a one-way ANOVA in order to test the effects of acetone used as fungicide solvent on the AM fungi growth parameters. Because no significant difference was observed between both treatments (result not shown), the impact of fungicides was compared solely to the $MSR^{acetone}$ control. The hyphal length of ERM, number of spores, and the number of anastomoses within the same network and between networks as well as the total number of anastomoses produced per hyphal length in the CC were analyzed with a one-way ANOVA followed by a Tukey Honest Significant Difference post hoc test. Specifically, the aov function of the multcomp R package (Hothorn et al. 2008) was used, where the experimental repetitions were introduced as random effect. No significant differences were observed between the experimental repetitions. A normal distribution was observed for the hyphal length of ERM, the number of anastomoses between networks and the total number of anastomoses and spores produced per hyphal length. To satisfy the model assumptions (normality and homoscedasticity of the residuals), however, the number of spores produced and the number of anastomoses within the same network were transformed using log10. All data were analyzed with R software version 4.1.2. and statistical significance was established at a 95% confidence level (i.e., when the P-value is below 0.05).

Results

No response variables were affected by pencycuron and flutolanil at either concentration in comparison with the $MSR^{acetone}$ control (**Fig. 27A–F**). In contrast, azoxystrobin at the highest concentration (2 mg L^{-1}) significantly reduced hyphal length, total number of spores, total number of spores per hyphal length, and total number of anastomoses within the same network or between different networks compared to the $MSR^{acetone}$ control and to azoxystrobin at the lowest concentration (0.02 mg L^{-1}) which never differed from the $MSR^{acetone}$ control (**Fig. 27A–E**). The number of anastomoses per hyphal length was not affected by azoxystrobin, whatever the concentration applied (**Fig. 27F**). A similar trend was observed in presence of fenpropimorph at the highest concentration (2 mg L^{-1}). In presence of fenpropimorph at 2 mg L^{-1}, the number of anastomoses within the same network or between different networks were significantly reduced as compared to the $MSR^{acetone}$ treatment and fenpropimorph at the lowest concentration (0.02 mg L^{-1}) (**Fig. 27C**, **D**). At both concentrations, the total number of anastomoses per hyphal length was significantly reduced by fenpropimorph as compared to the $MSR^{acetone}$ treatment (**Fig. 27F**). In addition, fenpropimorph at 0.02 mg L^{-1} significantly increased the total number of spores as compared to the $MSR^{acetone}$ and fenpropimorph at 2 mg L^{-1} (**Fig. 27B**). The hyphal length and total number of spores per hyphal length were not affected by the two concentrations of fenpropimorph (**Fig. 27A**, **E**).

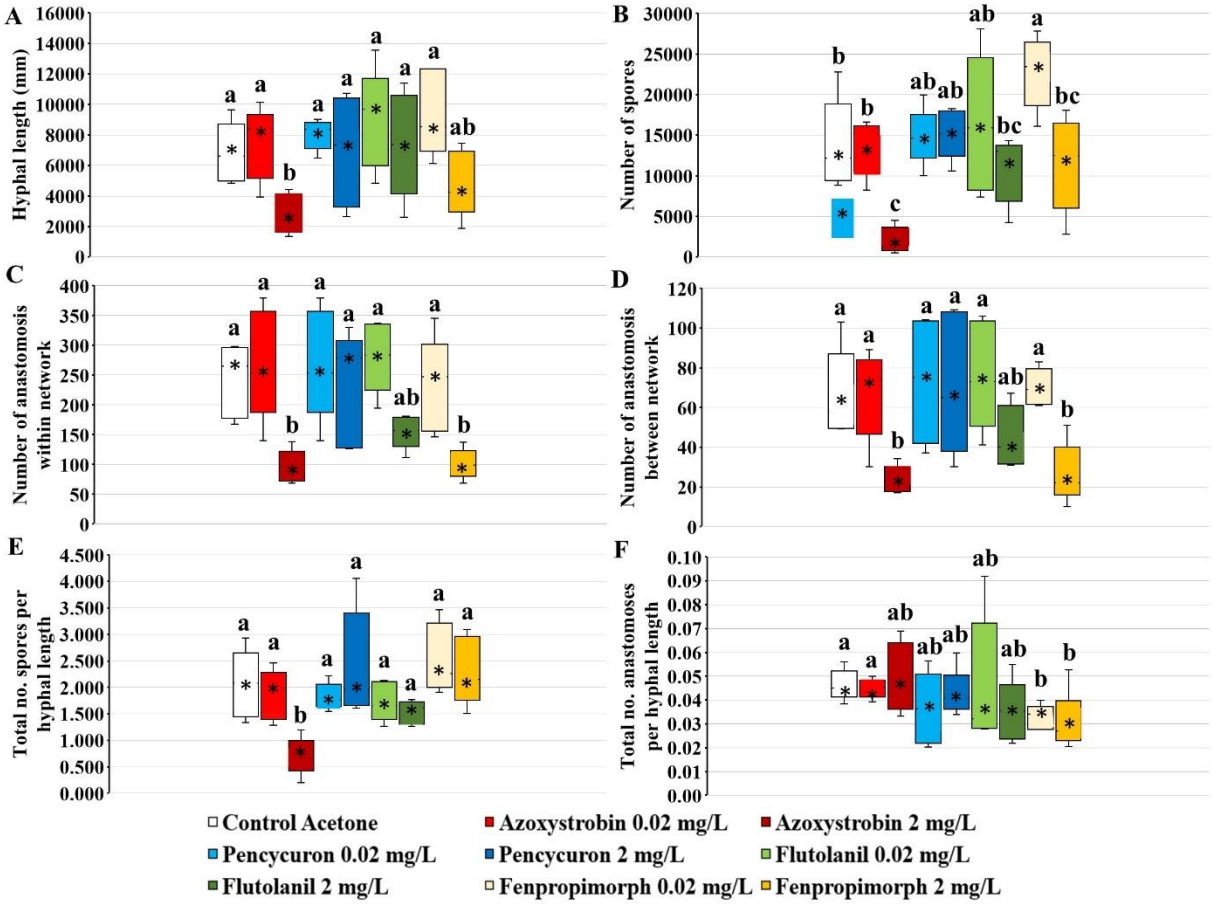

Fig. 27. Hyphal length (mm) (**A**), number of spores (**B**) and total number of spores per hyphal length (**E**), hyphal anastomosis within (**C**) and between (**D**) networks and total number of anastomoses per hyphal length (**F**) in extraradical mycelium (ERM) of *Rhizophagus irregularis* MUCL 41833 after 15 days of growth within the central compartment (CC) in the absence (control acetone) or in the presence of two concentrations (0.02 and 2 mg L^{-1}) of azoxystrobin, pencycuron, flutolanil, and fenpropimorph. Dots within boxes depict means (N = 5), boxes depict the interquartile range, and error bars depict ± standard error. For each parameter, the same letters indicate no significant difference between treatments, according to a one- way ANOVA followed by a Tukey Honest Significant Difference post hoc test ($P \leq 0.05$)

Discussion

The impact of pesticides on the anastomosis of AM fungi has been little studied. For instance, under *in vitro* conditions, Cardenas-Flores et al. (2011) reported a reduction in the anastomosis of *R. clarus* germlings in the presence of 10 mg L^{-1} of fenhexamid and de Novais et al. (2019a) noticed a decrease or null effect in anastomosis on germlings of *Funneliformis mosseae* lineages in growth chamber conditions with fenhexamid at 10 and 20 mg L^{-1} and benomyl at 0.17 and 1.07 mg L^{-1}. With chicory plants, de Novais et al. (2019b) noticed also that benomyl at 6.3 mg L^{-1} and fenhexamid at 20 mg L^{-1} decreased the ERM of *F. mosseae* but did not affect the anastomosis process.

In our study, differences were observed between active ingredients and concentrations of fungicides. Azoxystrobin and fenpropimorph at 0.02 mg L^{-1} did not affect the total number of anastomoses within and between networks, while at 2 mg L^{-1} a significant decrease was noticed for both parameters. This could be partly related to the decrease in total hyphal length (respectively 63% and 49% compared to the length at 0.02 mg L^{-1}) observed with both active

ingredients, reducing the probability for hyphae to anastomose. With fenpropimorph, however, the total number of anastomosis per hyphal length at 2 mg L^{-1} as well as 0.02 mg L^{-1} was significantly decreased, which was not the case with azoxystrobin, suggesting a more drastic effect of this active ingredient. These results complement the observations made by Rodriguez-Morelos et al. (2021) on the effects of both active ingredients at 2 mg L^{-1} on the hyphal healing mechanism (HHM) of *R. irregularis*. Those authors observed that azoxystrobin and fenpropimorph applied at 2 mg L^{-1} on injured hyphae drastically affected the mechanism of recognition between the cut extremities and therefore wound healing, while no impact was noticed at 0.02 mg L^{-1}. They suggested a disruption of the c These secretory vesicles have been suggested in AM fungi, although not conclusively demonstrated (de la Providencia et al. 2005).

In their study, de la Providencia et al. (2005) and Voets et al. (2006) suggested that fusion-competent hyphae of AM fungi exhibit remote sensing, resulting in growing hyphal tips (GHTs) initiation and/or redirection of growth to facilitate contact between participating hyphae. This was clearly shown for the HHM of *Gigaspora* and *Scutellospora* species and suggested in *Glomus*/*Rhizophagus* species (de la Providencia et al. 2005), supporting that the growth of GHTs or hyphae towards each other was due to elicitation of diffusible substance (i.e., secretory vesicles) and that this mechanism was led by one GHT (branch leader) or hyphae in a sequence of signaling-responses. It is not excluded, but must be firmly demonstrated, that in the presence of some fungicides (e.g., azoxystrobin and fenpropimorph), these attributes are "partially" lost, disrupting the directional memory, or resulting in growth arrest, thus reducing/annihilating the anastomosis process. That the number of anastomoses per hyphal length was affected by fenpropimorph at both concentrations, while this was not the case for azoxystrobin, tends to suggest that fenpropimorph was more detrimental to the signaling-response mechanisms and thus to anastomosis formation than azoxystrobin.

Our results with fenpropimorph further support studies conducted under *in vitro* culture conditions by Campagnac et al. (2008, 2009, 2010) and Zocco et al. (2008, 2011). Those authors demonstrated that this sterol biosynthesis inhibitor at doses equal or above 0.2 mg L^{-1} decreases ERM growth of *R. irregularis*. It is well known that fenpropimorph inhibits ergosterol biosynthesis (Debieu et al. 2000), which is the typical sterol in the vast majority of fungi but absent from AM fungi, while 24-methyl and 24-ethyl cholesterol are the two major sterol compounds in AM fungi (Fontaine et al. 2001; Olsson et al. 2003). Interestingly, Steinberg (2007) suggested that sterol-rich membrane domains, termed lipid rafts, might support hyphal tip growth by facilitating apical endocytosis and providing an apical scaffold that organizes the cytoskeleton. Therefore, the impact of fenpropimorph on anastomosis possibly might be attributable to its impact on secretory vesicles as mentioned above and/or to the inhibition of the metabolism of membrane components as sterols in hyphal tips. Curiously, a stimulatory effect on spore production was observed at the lowest dose of fenpropimorph compared to the control. It is difficult to speculate what mechanism might be involved but may indicate a stress response to fungicide exposure at small doses.

Azoxystrobin exerts its fungicidal action by blocking electron transport in the mitochondrial respiratory chain in fungi (Balba 2007). This drastically reduces energy production, thereby inhibiting growth of the fungus as was shown by the 63% decrease in total hyphal length and 84% reduction in spore production between the 0.02 and 2 mg L^{-1} concentrations in our experiment. This also resulted in a significant decrease in the total number of anastomosis within and between networks, while expressed in the number of anastomoses per hyphal length, no impact was detected. This suggested that this active ingredient has probably less impact than fenpropimorph on the remote sensing mechanism between hyphae. Neither pencycuron (a phenylurea highly specific to sensitive strains of *Rhizoctonia solani* – Young et al. 2012) nor flutolanil (a highly systemic phenyl-benzamide used against Basidiomycota pathogens – Zhao et al. 2019) affected mycelium growth and anastomosis of *R. irregularis* at either concentration. This corroborates the study of Rodriguez-Morelos et al. (2021) showing an absence of effects of these fungicides at the same concentrations on the HHM of *R. irregularis* and the work of Buysens et al. (2015), which noticed that flutolanil at 0.1 mg L^{-1}, did not affect ERM grown. Thus, the absence of impact suggests that the enzymes involved in cell division in hyphal tips or the respiration pathway in *R. irregularis* are different or less sensitive than those in *R. solani* or Basidiomycota pathogens, respectively.

In conclusion, this work reports for the first time the direct effects of fungicides on hyphal anastomosis between different networks of the same strain of *R. irregularis*, complementing results already obtained on anastomoses between hyphae within the same mycelial network. The effects vary with active ingredient and concentration, with the broad- spectrum fungicides azoxystrobin and fenpropimorph being the most detrimental at 2 mg L^{-1}. This concentration often is considered above field dosage for azoxystrobin. For example, Buysens et al. (2015) suggested that 0.75 mg L^{-1} concentration *in vitro* corresponds to 1500 g ha^{-1} (formulation Amistar) to control *R. solani* in potato. Zocco et al. (2011) suggested that fenpropimorph at 2 mg L^{-1} corresponds to 0.17 times the recommended dose for field application (formulation Corbel). Therefore, even if fenpropimorph appears more detrimental than azoxystrobin at the same concentration, its effects may be less marked in the field because of the lower dose of application. It is important to note that the impact of fungicides on AM fungi under *in vitro* culture conditions can only partially reflect field situations, where edaphic factors (e.g., soil physico-chemistry and microbial populations) determine fungicide degradation. *In vitro* culture, however, allows non-destructive investigation of the role of fungicides on AM fungi under conditions exempt of confounding effects. Therefore, both approaches (within field and *in vitro*) have complementary strengths to investigate and determine the role of fungicides on the non-target AM fungi. Our results support the hypothesis that fungicides may negatively affect anastomosis by altering the hyphal tips of AM fungi and may thus affect the ability of AM fungi to develop large networks of hyphae exploring and exploiting the soil to serve plants.

Conflict of interest

The authors declare no competing interests.

Author contributions

Stéphane Declerck conceived the study and provided resources. Victor Hugo Rodriguez-Morelos carried out the experimental work and wrote the manuscript. Victor Hugo Rodriguez-Morelos, Maryline Calonne-Salmon and Stéphane Declerck, revised the manuscript and approved the final version.

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Research results: PART II

Impact of azoxystrobin on phosphorus nutrition of mycorrhizal plants

Preface

The impacts of fungicides on P transport by AM fungi have been reported in a few studies. However, the accumulation of Pi in mycorrhizal plants has most often been evaluated destructively (i.e. at harvest), thus by quantification of P at a single time point, not considering short-term dynamics of Pi uptake. Therefore, the impact of azoxystrobin (one of the most widely used fungicides) on Pi uptake by mycorrhizal plants is unknown.

In chapters I and III, it was firmly demonstrated that azoxystrobin at 2 mg L^{-1} impacted drastically the HHM of *R. irregularis* and the total number of anastomosis formed within and between networks, while at 0.02 mg L^{-1} no effects were noticed. The concentration of 2 mg L^{-1} of a.i. used at the place of hyphal injury was probably above the recommended field dosage, while 0.02 mg L^{-1} was below. From these results, it is speculative to conclude that field recommended dosage may impact the fungus significantly. Therefore, an experiment was performed under a semi-hydroponic cultivation system to evaluate the impact of azoxystrobin on the Pi uptake dynamics of AM fungal-colonized potato plants under recommended field dosage to control *Rh. solani* in potato cropping system.

The objective of **chapter IV** was to investigate the dynamics of Pi uptake of potato plants associated or not to *Rhizophagus irregularis* MUCL 41833 in presence or absence of azoxystrobin at the concentration of 1.17 mg of fungicide per gram of perlite. We used a circulatory semi-hydroponic cultivation system connecting each plant to a nutrient solution under greenhouse conditions. Then, Pi depletion was measured at regular intervals in the nutrient solution flowing through the plant containers, thus indirectly assessing Pi uptake by the plants associated or not to the AM fungus.

Chapter IV

Azoxystrobin alters the dynamics of short-term phosphorus uptake of mycorrhizal potato plants associated to *Rhizophagus irregularis*

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Abstract

Azoxystrobin is widely used in crops against several fungal diseases but may have potential negative effects on soil microorganisms beneficial to plants such as arbuscular mycorrhizal (AM) fungi. To date, the impact of azoxystrobin has been studied on the growth and healing capacity of AM fungi, but not on the inorganic phosphorus (Pi) uptake dynamics.

The aim of the study was to investigate the impact of azoxystrobin on the dynamics of Pi uptake by potato plants associated or not to *Rhizophagus irregularis*. Additionally, fungal root colonization, plant growth, and P accumulation were assessed. A semi-hydroponic cultivation system was used with mycorrhizal (M) and non-mycorrhizal (NM) plants growing in presence/absence of azoxystrobin. The percentage of Pi depletion in the nutrient solution was determined by inductively coupled plasma atomic emission spectroscopy after 30 and 60 days of circulation. Whatever the presence/absence of the AM fungus, azoxystrobin decreased Pi uptake by plants after 30 days of growth. Conversely, after 60 days in absence of azoxystrobin, a larger Pi uptake was measured in the M plants, while the reverse was noticed in presence of azoxystrobin. The M plants were more affected by azoxystrobin than those growing in absence of the fungicide, suggesting a direct effect of azoxystrobin on the AM fungus and an indirect effect on the plant–fungus associates via a potential imbalance in the transfer of nutrients. Although it is difficult to extrapolate our results to field, fungicide toxicity on crops inoculated with selected AM fungi deserves to be explored.

Keywords

Arbuscular mycorrhizal fungi · Fungicide · Inorganic phosphorus · *Rhizophagus irregularis*, *Solanum tuberosum* · Strobilurin

Introduction

Strobilurins are fungicides widely applied in agricultural fields for decades (Bartlett et al. 2002). They are among the most largely sold synthetic chemicals because of their efficient germicidal activity and fast degradation during plant metabolism (Feng, Huang, et al., 2020). Generally considered fungicides with systemic activity, they affect mitochondrial respiration in various fungi belonging to the taxa Ascomycota, Basidiomycota, and Deuteromycota as well as the fungal-like Oomycota (Bartlett et al. 2002; Feng, Huang, et al. 2020).

Azoxystrobin, a fungicide within the strobilurins, is recommended for the control of potato diseases caused by *Rhizoctonia solani* Kühn (Djébali et al. 2014) and *Alternaria solani* Sorauer (Landschoot et al. 2017). However, soil application of this fungicide has been shown to impact nontarget soil bacteria, annelids, and symbiotic fungi, as arbuscular mycorrhizal (AM) fungi (Adetutu et al. 2008; Kovačević et al. 2021; Zhang et al. 2019). The review of Hage-Ahmed et al. (2019) reported that several studies conducted under contrasting environmental conditions showed that fungicides could impact AM fungi either directly by affecting, for example, root colonization or extraradical mycelium (ERM) development or indirectly by impacting the physiological responses of the host plant (Buysens et al., 2015; Diedhiou et al. 2004; Vuyyuru et al. 2018). For instance, several greenhouse studies have demonstrated that soil drenching with azoxystrobin inhibits root colonization or enzymatic activity of AM fungi (Diedhiou et al. 2004; Vuyyuru et al. 2018). Conversely, foliar application of azoxystrobin appeared less detrimental or did not affect AM fungal root colonization (Campos et al. 2015; Diedhiou et al. 2004; Hernández-Dorrego and Mestre-Parés 2010). Under *in vitro* culture conditions, it has been shown that azoxystrobin as the active ingredient (a.i.) alone or formulated (i.e., Amistar), used at concentrations above threshold values for the control of *R. solani*, affects spore germination and production, development of ERM, and root colonization of potato by the AM fungus *R. irregularis* (Buysens et al. 2015). Recently, the hyphal healing mechanism of two AM fungi (i.e., *R. irregularis* and *Gigaspora* sp.) grown *in vitro* was reported to be strongly affected at 2 mg L^{-1} of this fungicide (Rodriguez-Morelos et al., 2021). However, up to date, no study has focused on the impact of this fungicide on the ability of plants associated to AM fungi to take up more phosphorus (P) than non-associated plants.

Phosphorus is available to plants in its inorganic (Pi) form in soils, in which this element is poorly soluble and mobile (Javot et al. 2007). Interestingly, the AM symbiosis can increase the plant Pi uptake by extending the zone of root exploration, via the ERM network, to areas otherwise inaccessible (Ferrol et al., 2019). This Pi-enhanced nutrition can improve plant growth and health and therefore increase their tolerance to biotic (Diagne et al. 2020) and abiotic (Begum et al., 2019; Lenoir et al., 2016) stresses. Although the benefits provided by AM fungi have been demonstrated on potato crops (Alaux et al. 2020; Buysens et al., 2016; Chifetete and Dames, 2020; Hijri 2016; Yang et al. 2020), only a few studies have been conducted on the role of AM fungi on host Pi uptake in the presence of fungicides (Boatman et al. 1978; Gnekow and Marschner 1989; Hale and Sanders 1982; Kling and Jakobsen 1997; Schweiger and Jakobsen 1998; Sukarno et al. 1996; Zocco et al. 2011) and no one evaluated the dynamics of Pi uptake by mycorrhizal plants. Here, we aimed to compensate for this missing information by evaluating the dynamics of Pi uptake of potato plants associated or not to an AM fungus and grown in presence or absence of azoxystrobin. The study was conducted nondestructively under a circulatory semi-hydroponic cultivation system connecting each plant to a nutrient solution. Pi depletion was

measured at regular intervals in the nutrient solution flowing through the plant containers, thus indirectly assessing Pi uptake by the potato/AM fungus associates.

Materials and methods

Biological material

Potato plantlets (*Solanum tuberosum* L. var. Bintje; Station de Haute Belgique in Libramont, Belgium) were micropropagated in vitro every 5 weeks as described in Gallou et al. (2011). Seeds of maize (Troizi variety from Caussade®, France) and medic (*Medicago truncatula* L., cv. Jemalong A17 from the South Australian Research and Development Institute, Australia) were treated by bleach solution (8% active chloride) for 15 min and rinsed three times with sterilized (121°C for 15 min) deionized water (Calonne-Salmon et al. 2018).

Mass production of the AM fungus *R. irregularis* MUCL 41833 in greenhouse conditions.

One 13.2 L (56.5 × 36 × 6.5 cm) plastic tray containing sterilized (121 °C for 15 min) lava stone (DCM, Belgium) was inoculated with spores and colonized root pieces of the mass-produced AM fungus *R. irregularis* MUCL 41833 (see above). This inoculum was placed between two layers of the substrate. A second plastic tray was prepared with the same substrate without AM fungal inoculum. Maize plants were first planted for 4 months in the substrate for root colonization and ERM establishment of the AM fungus. The plants were then cut (only leaving roots in the substrate) to let space and light at the surface of the tray and *M. truncatula* seeds sown to produce mycorrhizal (M) and non-mycorrhizal (NM) donor plants. This plant indeed takes lower space and light. The trays were maintained under controlled conditions at 22°C/18°C (day/night), a relative humidity of 70%, a photoperiod of 16 h day^{-1}, and a photosynthetic photon flux of 120 µmol m^{-2} s^{-1} in greenhouse (**Figure 28**).



Fig. 28. Mass-production of *R. irregularis* MUCL 41833 in trays with medic (*Medicago truncatula*) and medic plants without AM fungal inoculum under greenhouse conditions.

Colonization of *S. tuberosum* plants

After 8 months of culture, four randomly selected medic plants in the M and NM trays were stained and microscopically observed to determine the percentage of total root colonization (%TRC; see method below). The %TRC reached a value of 95.5% ± 9.3% in M plants, whereas no AM fungal structures were detected in the roots of the NM plants. At that time, 80 potato plants (see above) were planted in the trays. After 2 months, the %TRC was estimated on four randomly selected potato plants in the M and NM trays. The %TRC of the M plants reached 55.5% ± 3.5% and the vesicular colonization reached 49.6% ± 4.9%, while no AM fungal structures were detected in the roots of the NM plants. Plants were considered as adequately colonized and were transferred to their individual container.

Preparation of azoxystrobin contaminated substrate

Perlite was washed and dried as described in detail by Garcés-Ruiz et al. (2017), before adding fungicides. For this, 32 g of perlite was soaked in 32 mL of acetone containing 0.56 mg L^{-1} azoxystrobin (Sigma–Aldrich, Inc., Darmstadt, Germany) in order to obtain 1.17 mg of fungicide per gram of perlite (i.e., 37.5 mg per container) (i.e., the azoxystrobin [AZO] treatment). A control acetone (CA) treatment was prepared by mixing 32 g of perlite with 32 mL of acetone without fungicide. A control water (CW) treatment without acetone and azoxystrobin was prepared by mixing the same amount of perlite with 32 mL of sterile deionized water. For each treatment, perlite was left to dry for 60 h in a chemical hood. Each container (see detailed container preparation in Garcés-Ruiz et al. 2017) was then filled with perlite. Finally, three non-vegetated (NV) containers per treatment with either azoxystrobin, acetone, or water were included in the design.

Experimental setup

After 2 months of colonization in the planting trays, the potato plants were carefully removed from their substrate and their roots were gently cleaned with deionized water. The M and NM potato plants (see above) were acclimatized during two weeks in the perlite substrate of the different treatments. The plants received 200 ml of Hoagland solution (90% P-impoverished solution or 6.245 mg L^{-1} - termed hereafter Hoagland low-P – **see composition in Annex III**) every 48 h.

The reduction of P was made to favor the root colonization by the AM fungus. Indeed, results demonstrated the increase in root colonization of wheat with *F. mosseae* by reducing the phosphorus concentration by 10 times in the Hoagland solution, in comparison with the normal P concentration and the P concentration reduced by 5 times (Mustafa et al. 2016).

Each plant was then placed individually in the containers of the AZO, CA, and CW treatments, and randomly disposed on flex foam supports (Garcés-Ruiz et al. 2017). Each container was wrapped with aluminum foil and the surface of the perlite was covered with black quartz (Dekoline, Belgium) to prevent algae development (Calonne-Salmon et al. 2018). Six containers were considered per treatment. The experiment thus consisted of a factorial combination of fungicide treatment (AZO, CA, or CW) and AM fungal inoculation (M or NM). Three non-vegetated (NV) containers per treatment (AZO, CA and CW) were also prepared as negative controls.

Monitoring of Pi depletion in Hoagland low-P solution and determination of P accumulation in plants

Prior to the Pi-depletion short-term experiment, a flushing was performed to establish an equal nutrient concentration in all containers. Each container received 200 ml of Hoagland^{-P} solution. The flow rate in the multichannel peristaltic pumps was 44 ml min^{-1}.

The circulatory system was established to 1 L glass bottles covered with aluminum foil and containing the Hoagland low-P solution. Twenty-five ml of the solution was sampled from each bottle before the start of the circulatory system [considered as time 0 (T0)] to determine the initial Pi concentration in the bottles. After flushing, the circulating system was then initiated at a speed of 7.5 ml min^{-1}. At defined intervals, 20 ml of the nutrient solution was sampled in Falcon tubes of 50 ml and subsequently stored at 4°C in the dark before Pi analysis by inductive coupled plasma atomic emission spectrophotometer (ICP-AES). The duration of the experiment was 60 days (**Figure 29**). At two defined times (i.e., day 30 [first dynamics of Pi uptake] and day 60 [second dynamics of Pi uptake]).

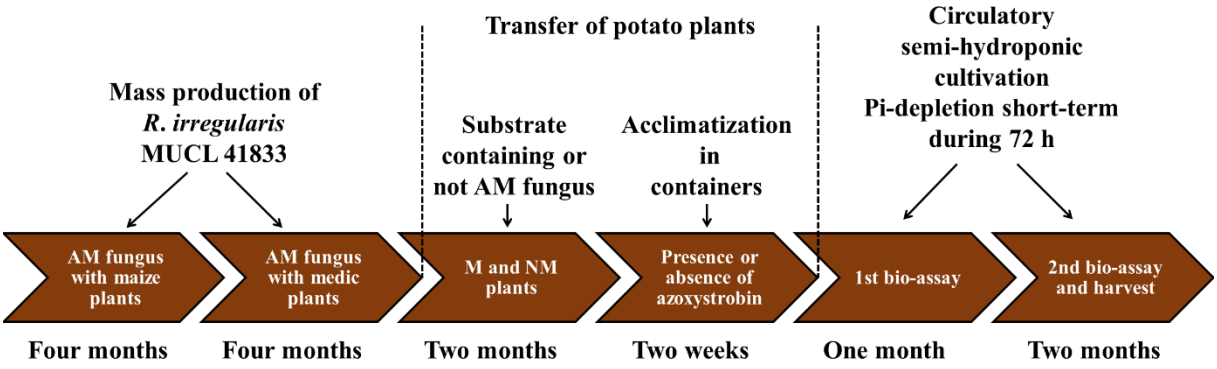

Fig. 29. Experimental process to evaluate the short-term Pi-depletion in mycorrhizal (M) and non-mycorrhizal (NM) potato plants growing in presence or absence of azoxystrobin using a circulatory semi-hydroponic cultivation system established under greenhouse conditions.

The percentage of Pi depletion in the Hoagland low-P solution was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) and standardized according to those obtained by their respective NV containers and Pi concentration at time 0 h

Two ml of each sample was diluted five times with ultrapure water (Millipore, France). The solution was then acidified with 20 ml of HNO_3 at 65% (Merck, Germany) and subsequently analyzed by ICP-AES (ICAP 6500, Thermo-Scientific, USA - see details below). Data obtained (in ppm) were converted in mg L^{-1}. The Pi depletion values obtained from the medium were standardized according to those obtained by their respective blanks and Pi concentration at time 0 h, following the formula below:

$$[P]_x = [P]_x \text{ quantified with ICP-AES at time T} + ([P]_{blank} \text{ at } T_0 - [P]_{blank} \text{ at T})$$

Where:

[P] = Pi concentration in the solution
X = sample
Blank = non-vegetated containers respective to the sample analyzed
T = time considered 1st bio-assay (12, 24, 48 and 72 h) and 2nd bio-assay (24, 48 and 72 h) after the start of the circulatory system.
T0 = 0 h before the start of the circulatory system

Net Pi uptake was determined from the depletion of Pi in the Hoagland low P solution circulating through the plant containers.

The P quantification in the Hoagland low-P solution after processing (digested solution) (see above) was analyzed by ICP-AES (ICAP 6500, Thermo-Scientific, USA). The ICP-AES is a specific method of emission spectroscopy, which excites atoms and ions with plasma equally, causing it to emit electromagnetic radiation at wavelengths characteristic of a particular element (Houck 2009). In addition, the ICP-AES quantifies the elemental concentration of the sample. For example, the ICP-AES uses high-energy plasma of argon gas to burn analytes quickly. The color that is emitted from the analyte is indicative of the elements present, and the intensity of the spectral signal shows the concentration of the elements in the sample (Thines et al. 2020).

The P content in liquid and in plant is then determined with axial viewing of the emitted radiation. A peristaltic pump is used to introduce the solutions into the ICP-AES at a flow rate of 1.5 ml min^{-1}. Operating parameters for the instrument include forward power 1150 W, coolant gas flow rate 12 L min^{-1}, auxiliary gas flow rate 1 L min^{-1} and nebulizer gas flow rate 0.6 L min^{-1}. Pi quantification is analyzed under a wavelength of 177.495 λ nm^{-1}. The limit of detection is <100 ppb (Garcés-Ruiz et al. 2017).

At the end of the experiment, the potato plants were harvested, the shoot, roots, and tubers fresh weights were estimated before drying in an oven at 50°C for 48 h to obtain the shoot dry weight (SDW) and root dry weight (RDW) and drying at 50°C for 96 h to obtain tuber dry weight (TDW). One g (fresh weight) of each root system was then used to evaluate AM fungal root colonization (see below).

After evaluation of dry weight, 100 mg of shoots and roots were ground separately in a grinder for P analysis. The ground material was then placed at 50 °C overnight. Each sample was transferred into an Erlenmeyer (Pyrex, United States) and 4 ml of HNO_3 65% was added for the digestion process. Erlenmeyers were covered with a watch-glass and placed on a heating plate at 60 °C overnight. After concentration at 120°C, samples were diluted with 3 ml HCl 38% (Merck, Germany), 1 ml HNO_3 65% and 10 ml of ultrapure water (Millipore, France). The solution was filtered with filter paper N°1 (Whatman, United Kingdom) in a 25 ml volumetric flask, before analysis. Phosphorus concentration was quantified in ICP-AES and data obtained in ppm was converted to mg kg^{-1} and content of P was determined according to the dry weights of shoots and roots.

Determination of AM root colonization

Before evaluation of RDW, 1 g (fresh weight) of each root system was collected to evaluate

AM fungal root colonization. The root samples were treated using a fungal staining technique described by Walker (2005) to evaluate the percentages of total root colonization (%TRC), arbuscular (%arbuscules), and spores/vesicles (%vesicles) colonization, using the magnified intersect method described in McGonigle et al. (1990). In total, 150 intersections per replicate were observed under microscope (Olympus BH2-RFCA, Japan) at 10–20× magnification to estimate the root colonization by the fungus.

Statistical analysis

Levene and Shapiro–Wilk tests were performed before the statistical analysis to demonstrate homogeneity of variance and normality of distribution, respectively. To achieve normality and homoscedasticity, data were log–10 (for dry weight, P concentration, and content) or arcsine (for root colonization rate) transformed. In our analysis, treatment CA was defined as the control group. Prior to the statistical analyses between the CA and AZO treatments, statistical analyses on the whole data were performed to compare both control treatments (i.e., CA–NM, CA–M, CW–NM, and CW–M treatments), in order to test the effects of acetone used as azoxystrobin solvent. In the analyses, no significant difference was observed between the CA and CW treatments (see Supporting Information); thus, only the CA treatment was considered to assess the effect of azoxystrobin on evaluated parameters.

The interaction between "AM fungal inoculation" and "fungicide treatment" on the percentage of Pi depletion over time was analyzed with a mixed model for repeated measurements, where "time" and a factor resulting from the mixture (= "groups") of "AM fun- gal inoculation" and "fungicide treatment" (= four conditions) were considered as fixed factors. When the interaction was significant, thus meaning that the effect of the factor "treatments" was according to the time, a Tukey Honest Significant Difference post hoc test was performed to focus on the effect of the factor "groups" at each time. The P concentration and content and the SDW, RDW, and TDW were subjected to a two-way ANOVA and followed by Tukey's post hoc test to compare the means between the treatments ([a] CA–NM vs. CA–M, [b] AZO–NM vs. AZO–M, [c] CA–NM vs. AZO–NM, [d] CA–M vs. AZO–M).

Data were analyzed with R software version 4.1.1. Finally, a one-way ANOVA was performed to test the difference in percentages of root colonization (i.e., %TRC, %arbuscules, and %vesicles) in the M plants growing in presence or absence of azoxystrobin. Data were analyzed using International Business Machines Corporation Statistical Package for the Social Sciences Statistics 27. For all analyses, statistical significance was set at a 95% confidence level (i.e., when the p-value is lower than 0.05).

Results

Dynamics of Pi depletion in the Hoagland low-P nutrient solution supplemented or not with azoxystrobin

The dynamics of Pi depletion in the Hoagland low-P solution was monitored during 72 h, at day 30 (first dynamics of Pi uptake; **Figure 30A**) and day 60 (second dynamics of Pi uptake; **Figure 30B**), as a ratio between the Pi concentration in the solution at selected timepoints and Pi concentration in the solution at time 0 h and expressed as a percentage of Pi (%Pi) remaining in the nutrient solution.

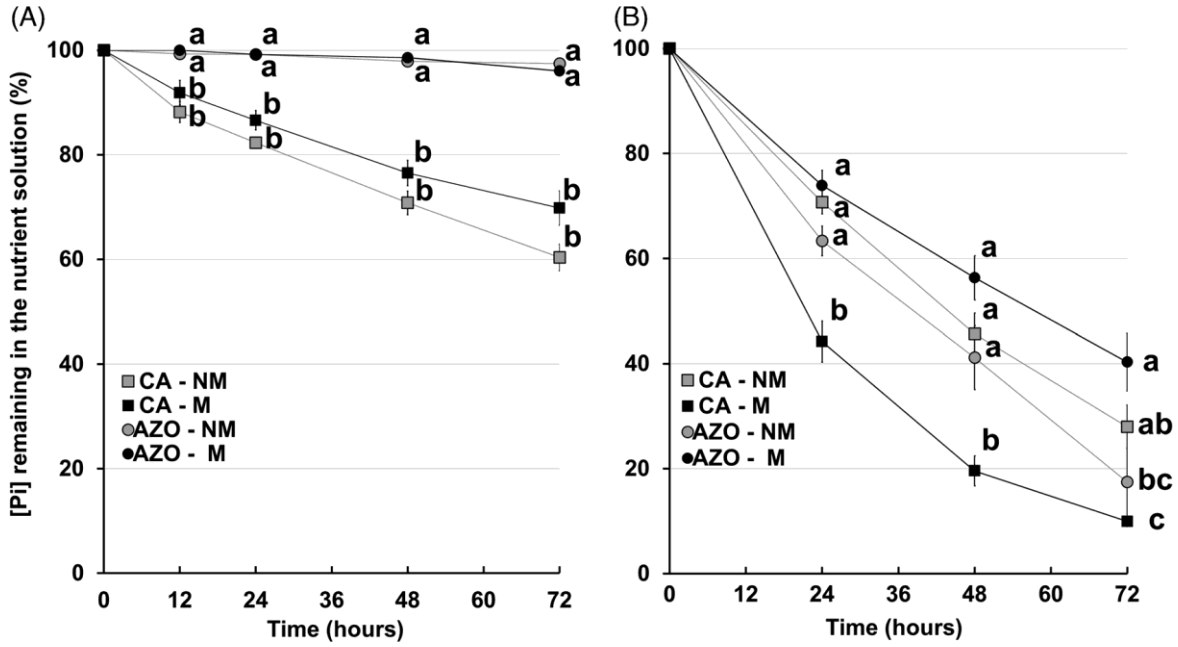

Fig. 30. Percentage of Pi concentration remaining in the Hoagland low-P solution after 72 h. The mycorrhizal (M) or nonmycorrhizal (NM) potato plants were grown **(A)** 30 and **(B)** 60 days, in perlite without azoxystrobin (CA) or containing 1.17 mg g^{-1} of azoxystrobin (AZO). Data are presented as means ($n = 6$) ± standard error at 30 days and means ($n = 5$ for CA–NM and AZO–NM; $n = 6$ for CA–M; $n = 3$ for AZO–M) ± standard error at 60 days. At each time of observation, means with the same lowercase letter did not differ significantly, according to the mixed model for repeated measurements and Tukey's post hoc tests ($p \leq 0.05$).

At day 30 (first dynamics of Pi uptake), the %Pi remaining in the nutrient solution was almost unchanged over the 72 h of measurement in the AZO treatments, while in the CA treatments it decreased over time, in presence as well as absence of the AM fungus. A significant interaction ($p < 0.001$) was noted between the factor "time" and "treatment," suggesting differences between the four treatments according to the time of analysis. Indeed, whatever the time of observation, the %Pi remaining in the Hoagland low-P solution was significantly higher in the AZO treatments as compared to the CA treatments, whether the plants are mycorrhizal or not. At each measurement, no differences in %Pi remaining in the nutrient solution was noticed between M and NM plants, in presence of the fungicide or in absence of the fungicide (**Figure 30A**). No significant differences were observed between the CA and CW treatments in absence or presence of AM fungus (result not shown).

At day 60 (second dynamics of Pi uptake), the %Pi remaining in the Hoagland low-P solution decreased over the 72 h of measurement, in presence as well as absence of the AM fungus. A

significant interaction ($p = 0.006$) was noticed between the factors "time" and "treatment" as in the first dynamics of Pi uptake by the plants.

Whatever the time of observation, a significant interaction was observed between "AM fungal inoculation" and "fungicide treatment" ($p = 0.013$, 0.014, and 0.005 at 24, 48, and 72 h, respectively). The %Pi remaining in the Hoagland low-P solution was significantly higher in the AZO–M treatment as compared to the CA–M treatment, while no difference was observed between the NM plants, irrespective of the presence or absence of the fungicide. In absence of the fungicide, more Pi was taken up by the M plants compared to the NM ones, whatever the time of observation ($p = 0.006$, 0.010, and 0.050 at times 24, 48, and 72 h, respectively). In contrast, in presence of the fungicide, the Pi uptake at 24 and 48 h was similar between the M and NM plants ($p = 0.350$ and 0.264, respectively), while at 72 h, the NM plants took up significantly more Pi than the M ones ($p = 0.019$) (**Figure 30B**).

A significant interaction ($p < 0.001$) was noted between the factors "time" and "treatment," suggesting differences between the control treatments according to the time of analysis (Figure S1). No significant difference was observed between the CA and CW treatments, while the %Pi remaining into the Hoagland low-P solution was significantly lower in the M plants as compared to the NM ones irrespective of the control treatment, whatever the time of observation ($p = 0.005$, 0.010, and 0.016 at 24, 48, and 72 h, respectively) (Figure S1).

Plant and fungal growth parameters

The impact of azoxystrobin on the development of the potato plants and their colonization by the AM fungus was evaluated by measuring the RDW, SDW, and TDW on one side and by estimating the root colonization by the fungus on the other side.

At harvest, a significant interaction between "AM fungal inoculation" and "fungicide treatment" was noticed for RDW and SDW ($p = 0.003$ and 0.023, respectively) (**Figure 31 A**,**B**). Indeed, the NM plants had significantly higher RDW and SDW compared to the M ones in the AZO treatment, according to the Tukey test ($p = 0.003$ and 0.040 for RDW and SDW, respectively).

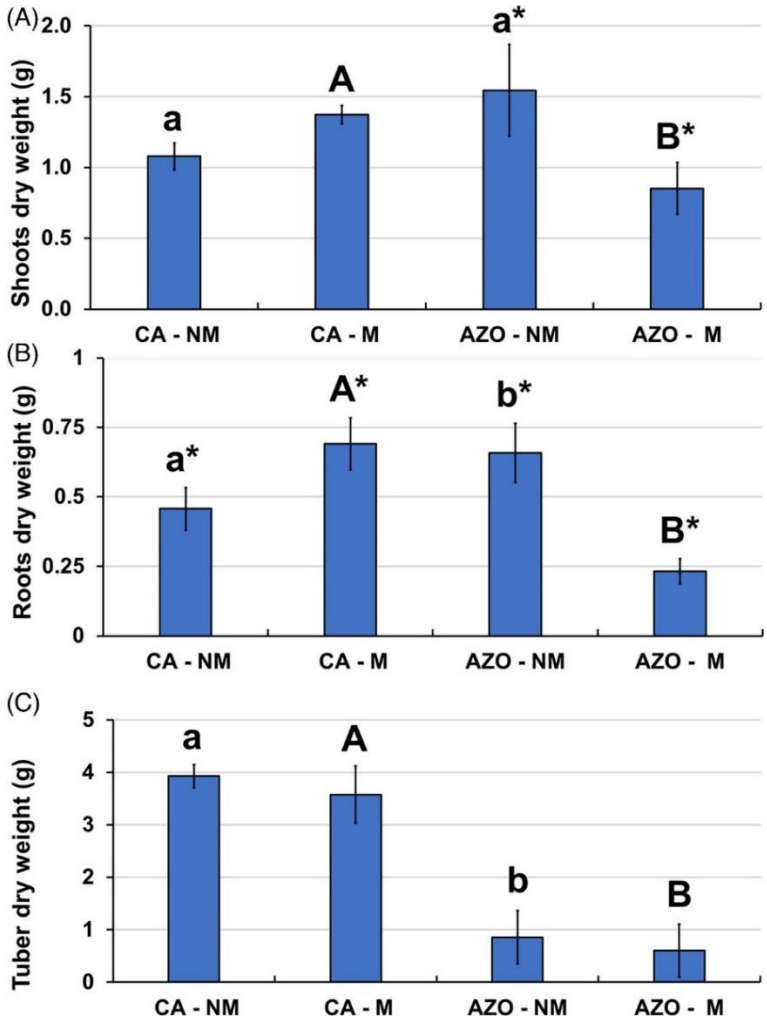

Fig. 31. Effect of azoxystrobin on the (**A**) shoot dry weight (SDW), (**B**) root dry weight (RDW), and (**C**) tuber dry weight (TDW) of mycorrhizal (M) and nonmycorrhizal (NM) potato plants at day 60 of growth in perlite without azoxystrobin (CA) or containing 1.17 mg g−1 of azoxystrobin (AZO). Data are presented as means (n = 5 for CA–NM and AZO–NM; n = 6 for CA–M; n = 3 for AZO–M) ± standard error. The data were analyzed by a two-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Different lowercase letters indicate significant differences between CA–NM and AZO–NM; different uppercase letters indicate significant differences between CA–M and AZO–M, and the presence of * indicates a significant difference between M and NM plants for each fungicide treatment.

In contrast, in the CA treatment, the RDW of the M plants ($p = 0.050$) but not the SDW ($p = 0.237$) was significantly in the NM treatments for SDW, with the exception of RDW that was significantly larger in presence of azoxystrobin. The presence of fungicide significantly affected the TDW ($p < 0.001$). Irrespective of the AM fungal inoculation, the application of azoxystrobin reduced the TDW of the plants in the AZO treatment compared to the plants in the CA treatment ($p = 0.009$ and 0.003 for the M and NM plants, respectively). For the control plants grown in the perlite nontreated or treated with acetone, a significant interaction between "AM fungal inoculation" and "control treatment" was noticed for RDW ($p = 0.011$), while SDW and TDW were not impacted ($p = 0.254$ and 0.513, respectively) (Figure S2). The RDW was not impacted in the CA and CW treatments irrespective of the presence or absence of the AM fungus. In contrast, the M plants had significantly higher RDW compared to the NM ones irrespective of the control treatment ($p = 0.015$ and 0.013 for the CW and CA treatments, respectively).

Root colonization of the potato plants in the containers was estimated at day 60. The plants in the AZO treatments had a lower ($p = 0.001$) %TRC, %vesicles, and %arbuscules colonization compared to those in the CA treatment (**Table 11**). No significant difference was observed in the root colonization between the CA and CW treatments (result not shown).

Table 11. Percentages of total root colonization (%TRC), spores/vesicles (%vesicles), and arbuscules (%arbuscules) root colonization of potato plants at day 60 of growth in perlite without azoxystrobin (CA) or containing 1.17 mg g^{-1} of azoxystrobin (AZO)

Treatments	%TRC	%vesicles	%arbuscules
CA	74.1 ± 9.5 a	72.9 ± 13.6 a	36.6 ± 8.8 a
AZO	8.7 ± 1.8 b	2.9 ± 0.8 b	2.5 ± 0.5 b

Note: Data are presented as means (n = 6 for CA and n = 3 for AZO) ± standard error. Data in a column with different lowercase letters are significantly different according to one-way ANOVA ($p \leq 0.05$).

P concentration and content of potato plants

The P accumulation in shoots and roots of the M and NM potato plants was measured at day 60 of growth in perlite containing or not azoxystrobin (**Table 12**).

Table 12. Effect of azoxystrobin on the phosphorus concentration (mg g^{-1} dry weight) and content (mg $plant^{-1}$) of mycorrhizal (M) and nonmycorrhizal (NM) potato plants at day 60 of growth in perlite without azoxystrobin (CA) or containing 1.17 mg g^{-1} of azoxystrobin (AZO).

	P concentration (mg g^{-1} dry weight)		P content (mg $plant^{-1}$)		
Treatments	**shoots**	**roots**	**shoots**	**roots**	**Total**
AM fungal inoculation					
NM	5.7 ± 0.5	1.7 ± 0.1*	5.0 ± 0.7	0.6 ± 0.06*	5.6 ± 0.7
M	5.3 ± 0.6	2.5 ± 0.2*	4.2 ± 0.6	0.9 ± 0.1*	5.1 ± 0.7
Fungicide treatment					
CA	5.6 ± 0.4	2.0 ± 0.2	5.0 ± 0.4	0.8 ± 0.1	5.8 ± 0.5
AZO	5.5 ± 0.7	2.2 ± 0.2	4.1 ± 0.9	0.6 ± 0.07	4.7 ± 1.0
AM fungal inoculation * Fungicide treatment					
CA – NM	6.3 ± 0.8a	1.5 ± 0.2a*	4.9 ± 0.7a	0.4 ± 0.3a*	5.3 ± 0.7a
CA – M	5.0 ± 0.3A	2.4 ± 0.2A*	5.1 ± 0.5A	1.2 ± 0.1A*	6.3 ± 0.6A
AZO – NM	5.2 ± 0.7a	1.9 ± 0.2a	5.2 ± 1.2a	0.8 ± 0.06b*	5.9 ± 1.3a
AZO – M	6.1 ± 1.8A	2.7 ± 0.5A	2.7 ± 0.4A	0.4 ± 0.1B*	2.9 ± 0.3B
	P value				
AM fungal inoculation	0.599	**0.003**	0.278	**0.011**	0.409
Fungicide treatment	0.773	0.145	0.103	0.163	0.086
AM fungal inoculation * Fungicide treatment	0.302	0.445	0.096	**0.001**	**0.037**

Note: Data are presented as means (n = 5 for CA–NM and AZO–NM; n = 6 for CA–M; n = 3 for AZO–M) ± standard error. Data were analyzed by a two-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). In a column, different lowercase letters indicate significant differences between CA–NM and AZO–NM, different uppercase letters indicate significant differences between CA–M and AZO–M, and an asterisk (*) indicates a significant difference between M and NM plants for each fungicide treatment, according to the Tukey's post hoc test ($p \leq 0.05$). The absence of any sign for the factors "AM fungal inoculation" or "fungicide treatment" indicates the absence of differences between treatments. Values in bold letters indicate -p-values ≤ 0.05.

Whatever the fungicide treatment, the P concentration and con- tent in shoots remained similar, while in roots both parameters were significantly impacted by the presence of the AM fungus ($p = 0.003$ and 0.011, respectively). Indeed, in the roots of the M plants, a larger P concentration and content were noticed compared to the NM ones ($p = 0.028$ and $p < 0.001$, respectively). This was particularly true for the plants grown in absence of the fungicide. The interaction between the factor "AM fungal inoculation" and "fungicide treatment" impacted the P content of roots and total plant ($p < 0.001$ and $p = 0.037$, respectively). In the CA treatment, the roots in the NM plants accumulated significantly less P than the M ones ($p < 0.001$), whereas in the AZO treatment the reverse was observed ($p = 0.028$). Plants associated or not to the fungus presented similar P content in the CA treatment ($p = 0.851$). On the contrary, the P content in the plants established in the AZO–M treatment was significantly lower as compared to the plants in the CA–M treatment ($p = 0.045$). For the control plants grown in the perlite nontreated or treated with acetone, only the fac- tor "AM fungal inoculation" impacted the P concentration of shoots and roots ($p = 0.004$ and 0.008, respectively) (Table S1). Indeed, the NM plants accumulated significantly more P in shoots than the M ones ($p = 0.018$) in the CW treatment. In contrast, the M plants accumulated significantly more P in roots than the NM ones ($p = 0.010$) in the CA treatment. Similarly, the AM fungal inoculation impacted P content in roots ($p = 0.001$). The M plants had significantly higher P content com- pared to the NM ones irrespective of the control treatment ($p = 0.005$ and 0.001 for the CW and CA treatments, respectively).

Discussion

In the present study, we report for the first time the impact of azoxystrobin on the short-term dynamics of Pi uptake of potato plants colonized by the AM fungus *R. irregularis* MUCL 41833, using a semi- hydroponic cultivation system connecting each plant to a nutrient solution circulating through a substrate containing or not azoxystrobin. The Pi uptake by M and NM potato plants was evaluated indirectly by quantifying the dynamics of Pi depletion in the Hoagland low-P solution.

In presence of azoxystrobin, a strong difference in Pi uptake by the potato plants was noticed between day 30 (first dynamics of Pi uptake) and day 60 (second dynamics of Pi uptake) with nearly no Pi uptake at day 30 conversely to day 60, where 80% and 60% of Pi were taken up by the NM and M plants, respectively. This noticeable difference is probably related to the dissipation half-life of this compound in soil/substrate, which depends on various environmental factors. Indeed, under field conditions, the half-life of azoxystrobin is estimated to about 14 days under light (photodegradation), whereas it remains around 8–12 weeks in the absence of light and under aerobic conditions (Feng, Zhang, et al. 2020) and even until 301 days in field (Edwards et al. 2016). Microbial degradation of strobilurins has also been reported in the literature. For instance, *Cupriavidus* sp. CCH2 and *Rhodanobacter* sp. CCH1 (two bacterial strains) were reported to use azoxystrobin as unique carbon source for growth (Howell et al. 2014) and more recently, Feng, Zhang, et al. (2020) noticed that *Ochrobactrum anthropi* SH14 increased the azoxystrobin dissipation in sterile and nonsterile soils as compared with soils in absence of the bacteria. Under the current experimental conditions, the degradation pathway of azoxystrobin was unknown. The nutrient solution circulating in presence of azoxystrobin was maintained in the dark, although it circulated from bottle to plant in transparent tubes, and the presence of microorganisms in the bottles containing the plants is not to exclude. Despite the lack of exact knowledge of the prevailing light and microbial conditions in our system, the dissipation of azoxystrobin was presumably higher at day 60 compared to day 30, possibly lowering the toxicity threshold to a level allowing the plant alone or associated to the AM fungus to take up Pi.

At day 60 (second dynamics of Pi uptake), an interaction between the AM fungus and the fungicide was noticed. Indeed, in absence of azoxystrobin, a higher uptake of Pi by the M potato plants was observed at the three timepoints, in link with a higher P concentration and content measured in roots but not in shoot. This significant increase in Pi uptake by the plant–AM fungus associates in absence of fungicide has been widely documented (Ferrol et al., 2019), and for potato this was supported by the recent observations of Liu et al. (2018) and Yang et al. (2020). Curiously, in presence of azoxystrobin, the Pi uptake by the M and NM potato plants remained similar at 24 and 48 h, while after 72 h of solution circulation the M plants took up significantly less Pi than the NM ones, possibly explaining the lower P content in the roots of the M potato plants as compared to the NM ones. As reviewed by Hage-Ahmed et al. (2019), strobilurins could induce positive growth-promoting effects in plants via changes on the plant hormonal balance, which might alter the interaction between AM fungi and their host plant. The present decrease in Pi uptake by the M plants is most likely due to physiological perturbations of the AM fungus and possible trade-off between the potato benefits (gain in P and stress alleviation) relative to the cost in carbon resulting from the colonization by the AM fungus. Indeed, under our experimental conditions, root colonization was considerably reduced by azoxystrobin, with, respectively, 25- and 15-times fewer vesicles/spores and arbuscules in the roots of plants grown in presence of the fungicide compared to those grown in absence of azoxystrobin. This observation corroborates earlier studies conducted in the greenhouse showing that soil drenches with this fungicide reduced the root colonization of sugarcane (Vuyyuru et al. 2018), potato (Alarcón et al. 2022), and maize (Diedhiou et al. 2004) by *Glomeraceae* members, clearly demonstrating the marked impact of this strobilurin, probably on the similar target site as for pathogenic fungi (i.e., the respiratory electron transfer within mitochondria [Bartlett et al., 2002; Feng, Huang, et al. 2020.]). Interestingly, foliar application of azoxystrobin (Campos et al. 2015; Diedhiou et al. 2004; Hernández-Dorrego and Mestre-Parés, 2010) or kresoxim-methyl, another strobilurin fungicide (Diedhiou et al. 2004), did not impact AM fungal root colonization, probably because of the low uptake by the leaves combined to a low or absent phloem mobility of these fungicides (Bartlett et al. 2002). Our results also complement the observations made under *in vitro* culture conditions on the detrimental impact of azoxystrobin on the development of AM fungi (Buysens et al. 2015; Rodriguez-Morelos et al. 2021). Indeed, Buysens et al. (2015) demonstrated a reduction in spore production and mycelial growth of the same AM fungus grown in presence of 0.1 mg L^{-1} of Amistar (formulated azoxystrobin), while root colonization of potato was affected at higher concentrations (i.e., 1 and 10 mg L^{-1}). More recently, Rodriguez-Morelos et al. (2021) observed that this fungicide at 2 mg L^{-1} a.i. strongly inhibited the hyphae healing mechanism in the ERM of *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331 associated with Ri T-DNA transformed chicory roots. Therefore, azoxystrobin applied as soil drench could impact directly the AM fungal growth and the symbiosis functioning.

Notable differences were also noticed on the effects of azoxystrobin on M and NM plant growth parameters. Indeed, in the NM treatments, the SDW of the plants grown in the presence or absence of azoxystrobin remained similar, and RDW as well as P content in roots even increased significantly with azoxystrobin. As reported by Fritz et al. (2022), plant metabolism could be improved after application of this strobilurin fungicide, via an increase in chlorophyll content, photosynthetic activity, stomatal aperture, water consumption, plant antioxidant activity, accumulation of phenolic compounds as flavonols and hydrocinnamic acids, and so forth (Fritz et al. 2022; Schiavon et al. 2021). This observation was reversed in the M treatments, in which plant SDW and RDW were significantly lower when azoxystrobin was introduced to the culture substrate compared to the absence of the fungicide. Moreover, in presence of azoxystrobin, the SDW and RDW of the M plants were significantly lower

compared to the NM plants. These observations corroborate a number of other studies conducted with fungicides applied as soil drench. For instance, in onion plants inoculated with Rhizophagus intraradices, Funneliformis caledonius, and *Glomus* sp., soil application of benomyl at the recommended field dosage markedly decreased fungal and plant growth compared to M plants in absence of fungicide (Sukarno et al. 1993), while no impact was noticed on the growth of the NM plants. A plant growth suppression was observed in cotton seedlings inoculated with F. mosseae after soil drench with etridiazole in comparison with NM plants grown in absence of fungicide (Pattinson et al., 1997). Finally, a drastic reduction in growth was noticed in onion and leek plants associated with *R. intraradices* and *Rhizophagus fasciculatus* under increasing doses of benomyl and metalaxyl applied to soil compared with M and NM plants grown in absence of these fungicides (Jabaji-Hare and Kendrick 1987; Manjunath and Bagyaraj 1984). Both authors hypothesized that each AM fungus behaved differently to fungicides application. They suggested that the fungicides decreased root colonization and reduced or eliminated the beneficial effects of AM fungi, altering plant growth and P nutrition. The present results highlight a greater impact of azoxystrobin on the growth of the M plants suggesting that the AM fungus was unable to mitigate the impact of this fungicide. This was possibly linked to a direct impact of the fungicide on root colonization (the %TRC decreased by 65.4% in plants grown with azoxystrobin) and Pi transport functions of the fungus counterbalanced by a higher relative transport of carbon from the plant to the AM fungus.

Curiously, both M and NM plants presented a similar TDW in presence or absence of azoxystrobin, while a strong decrease (76.9% and 83.3% in the M and NM treatments, respectively) was noticed in the TDW of the plants when the fungicide was applied in the perlite substrate compared to those grown in absence of the fungicide. This was supported by Alarcón et al. (2022) that demonstrated in a pot experiment that soil applied with azoxystrobin (20% [w/v]) and isopyrazam (12.5% [w/v]), a broad-spectrum foliar fungicide belonging to the chemical class of ortho-substituted phenyl amides (as formulation ReflectXtra), impacted differently the tuber weight of two genotypes (VR808 and CB2011-509) of potato associated or not with *Claroideoglomus claroideum* and *Claroideoglomus lamellosum*. In presence of the fungicides, the tuber weight was lower in VR808 plants associated with each AM fungus as compared to M plants in absence of fungicide, while no differences were observed with NM plant in presence of fungicide. In the same study, the reverse was noticed with CB2011-509 plants. These authors hypothesized that the interactions between fungicide and AM fungi could be influenced by potato geno type with different phenolic compounds and antioxidant activity in tubers. Our results on Pi uptake and plant growth tend to support a functional alteration of the symbiosis relative to the cost in terms of nutrients exchanged with the AM fungus based on the availability of nutrients in the environment or the demand of Pi by the host plant.

To conclude, this study highlighted for the first time the effects of azoxystrobin on the short-term dynamics of Pi uptake by mycorrhizal potato plants grown in a semi-hydroponic cultivation system. The plants colonized by *R. irregularis* were more impacted by the fungicide than those growing in the absence of the symbiont, suggesting both a direct effect of azoxystrobin on the fungus (its capacity to absorb and transport Pi to the plant) and an indirect effect via a probable imbalance between the Pi transported from the AM fungus to the plant in rule of C transported from the host to the AM fungus. The dosage of azoxystrobin used in the study was similar to the field dosage (i.e., 1500 g a.i. ha^{-1}) (https://fytoweb.be/fr/autorisations accessed on May 3, 2021) recommended against *R. solani* in potato cropping systems. However, it is too early to state that this recommended dosage will significantly affect the plant–AM fungus associates in the field, because under field conditions many factors may affect the

fate of the fungicide (e.g., physicochemical characteristics of the soil, presence of microorganisms degrading the fungicides, etc.) and thus the concentration of the active substance to which the AM fungi are exposed (Hage-Ahmed et al. 2019; Rivera-Becerril et al. 2017). Importantly, in the field and in contrast to the present study, plants are growing in the presence of diverse assemblages of AM fungi (Verbruggen and Kiers 2010), some of which may be less affected by chemicals and therefore colonize plants, although recent studies have confirmed that continuous fungicide application decreases AM diversity in agricultural soils (Edlinger et al. 2022; Henning et al. 2018; Ren et al. 2020). From the present study, it is therefore difficult to extrapolate the results to the field. Further studies are therefore needed to assess the effects of fungicides on crops inoculated with selected AM fungi in order to arrive at a rational management of fungicides to control fungal pathogens with minimal impact on the beneficial microflora.

Conflict of interest

The authors declare no competing interests.

Author contributions

Victor Hugo Rodriguez-Morelos: carried out the experimental work, data collection, analysis and interpretation, drafting the work, commentaries corrections, final approval, and agreement with all aspects of the work. Maryline Calonne-Salmon: Data collection and interpretation, draft work commentaries and corrections, final approval and agreement with all aspects of the work. Stéphane Declerck: Substantial contributions to the conception and design of the experiments, interpretation of the data, draft corrections final approval and agreement with all aspects of the work.

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GENERAL DISCUSSION

Nowadays, pesticides play an important role in agricultural production because they can reduce losses caused by pests, diseases and weeds and thus increase the yield and quality of food quality (Tudi et al. 2021). However, the use of pesticides also has adverse consequences, including increased risks to human health and proven toxicity to non-target organisms (Kalyabina et al. 2021), some of which (e.g., pollinators, aquatic life, soil microorganisms) are crucial for the functioning of ecosystems (Chagnon et al. 2015). Among these are the arbuscular mycorrhizal (AM) fungi. These symbiotic soil microorganisms colonize the roots of most important agricultural crops, improving water and minerals (especially phosphorus) supply to their hosts (Begum et al. 2019; Hou et al. 2021). They develop extensive extraradical mycelium (ERM) networks extending tens of cm beyond the soil nutrient depletion zone surrounding plant roots, thereby enhancing nutrients uptake (Ferrol et al. 2019). A crucial mechanism in the ERM formation is hyphal anastomosis which is the fusion between branches of the same or different hyphae, which includes cell wall degradation and plasma membrane fusion (Giovannetti et al. 1999).

In the last four decades, several studies have reported the impact of fungicides and herbicides on AM fungi, with contrasting results reported (Trappe et al. 1984; Ellouze et al. 2008; Hage-Ahmed et al. 2019; Sweeney et al. 2022; Pagano et al. 2023). However, the effect of these pesticides on the anastomosis process has been scarcely addressed and no study, to our knowledge, has reported the effects of pesticides on the hyphal healing mechanism (HHM) or the capacity of AM fungi to form anastomoses between networks of the same AM fungal isolate.

Impact of pesticides on anastomosis and HHM in AM fungi having contrasting life history strategies

In the first part of the thesis, the studies were conducted under strict *in vitro* culture conditions with root organs or whole plants. These systems offer several advantages: (1), mostly, the absence of any confounding effects caused by unwanted microorganisms (e.g., bacteria, fungal pathogens), contaminants or environmental factors (e.g., soil physico-chemical parameters) (Buysens et al. 2015) and (2) the easy non-destructive time-course observations and imaging of growth and mycelium architecture. For example, spore germination (Dodd and Jeffries 1989; Venedikian et al. 1999; Chiocchio et al. 2000; Gong et al. 2014), mycelium development (Zocco et al. 2008; Buysens et al. 2015), root colonization (Wan et al. 1998; Campagnac et al. 2008, 2009; Calonne et al. 2010, 2012), anastomosis formation (Cardenas-Flores et al. 2011), biosynthesis pathway (Oger et al. 2009; Campagnac et al. 2010; González-Guerrero et al. 2010; Calonne et al. 2012) and transport of phosphorus from fungus to plant (Zocco et al. 2011) have been investigated *in vitro* in presence of different types of fungicides. Noticeably, *in vitro* culture systems also present some drawbacks and may only partially reflect field situations. Indeed, several factors may affect the fate of the pesticides (e.g., physicochemical characteristics of the soil, presence of microorganisms degrading the fungicides, etc.) (Zocco et al. 2008; Buysens et al. 2015; Rivera-Becerril et al. 2017; Hage-Ahmed et al. 2019). Futhermore, in the field and in contrast to *in vitro* systems, AM fungi form ERM that colonize and connect roots of many plants even if they belong to different species, resulting in a common mycorrhizal network (Begum et al. 2019). Notwithstanding these remarks, our results support a number of studies conducted in the past years using the same *in vitro* cultivation systems. Indeed, in the absence of pesticides, *R. irregularis* and *Gigaspora* sp. showed a great ability to repair injured hyphae, as previously reported by de la Providencia et al. (2005, 2007), and *R. irregularis* to form anastomoses within and between mycelial networks, as shown by Voets et

al. (2006). Therefore, the imaging approach used in the present thesis was adequate to study the HHM and anastomosis in the presence of pesticides.

We first evaluated the impact of four major fungicides (azoxystrobin, pencycuron, flutolanil, and fenpropimorph) (**Chapter 1**) and of glyphosate, the world most widely used herbicide (**Chapter 2**) on the HHM in two AM fungi (*Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833) having contrasting LHS. Then, in **Chapter 3**, we evaluated the impact of the same fungicides as in **Chapter 1** on the anastomosis formation within and between networks of *R. irregularis* MUCL 41833.

Remarkably, azoxystrobin (at high concentration – i.e., 2 mg L^{-1}) markedly impacted the HHM in both AM fungi. Therefore, our results complement those obtained by Buysens et al. (2015), suggesting that the direct application in the culture medium of azoxystrobin at concentrations equal or above to 0.1 mg L^{-1} (a.i. or formulated product) affects *R. irregularis* 41833 growth *in vitro*. Although, the high concentration used (2 mg L^{-1}) was probably above the recommended field dosage. For example, Buysens et al. (2015) suggested that 0.75 mg L^{-1} (formulation Amistar) is the threshold measured value to control *Rhizoctonia solani* under *in vitro* conditions. The detrimental negative impact of azoxystrobin at 2 mg L^{-1} on the contact and fusion of GHTs of both strains could possibly been attributed to the perturbation of apical vesicular bodies (AVP) in the hyphal tip which are known to play key roles in hyphal growth, orientation, branching and morphogenesis in fungi (Steinberg 2007). Indeed, a perturbation of these structures by chemicals could explain the lack of recognition between GHTs in *R. irregularis* and *Gigaspora* sp as previously suggested by de la Providencia et al. (2005, 2007).

Conversely, flutolanil and pencycuron did not impact the HHM, at the concentrations and AM fungi tested. The absence of impact of flutolanil and pencycuron suggests that the enzymatic metabolisms of the respiration and cell division in AM fungi are probably less sensitive to fungicides than those of Basidiomycota or *Rhizoctonia* strains, respectively. This corroborates partially the study of Buysens et al. (2015). These authors noticed that flutolanil at 0.1 mg L^{-1} a.i. did not affect ERM development, while root colonization decreased at 1 mg L^{-1} a.i. They suggested that flutolanil was more detrimental to the intraradical structures (hyphae and arbuscules) than ERM structures because it is an inhibitor of the succinate dehydrogenase and has a high systemic activity. In addition, the two concentrations tested (i.e., 0.02 and 2 mg L^{-1}) could be considered lower or higher respectively to the recommended field dosage (0.09 mg L^{-1}) calculated *in vitro* (Buysens et al. 2015). Futhermore, Buysens et al. (2015) noticed that pencycuron at 0.01 mg L^{-1} a.i. did not affect the ERM development or root colonization. To the contrary, at the concentration of 0.5 mg L^{-1} a.i. root colonization of *R. irregularis* was reduced. Thus, our results suggest that pencycuron at 0.02 and 2 mg L^{-1} had no noticeable effects on the HHM, while higher concentrations above the recommended dose calculated *in vitro* (0.25 mg L^{-1}) may affect other processes (e.g., spore germination, root colonization) of AM fungal life cycle.

Fenpropimorph stimulated the emission and contact of growing hyphal tips (GHTs) of *R. irregularis* at low concentration (at 0.02 mg L^{-1}) and inhibited HHM at high concentration (i.e., 2 mg L^{-1}), while no effect was observed at both concentrations on *Gigaspora* sp. These results support a differential sensitivity of AM fungus to the presence of fenpropimorph. It is not excluded that these differences may be related to the sterol composition in fungal membrane of both strains. Additionally, Zocco et al. (2011) suggested that fenpropimorph at 2 mg L^{-1} could correspond to 0.17 times the recommended values for field application (formulation Corbel) calculated *in vitro*. These results do not exclude a potential impact of these fungicides on the development of *R. irregularis* under field conditions following soil application. Although, it is still speculative to affirm that recommended dosages may impact AM fungi in the field

significantly. Our results support *in vitro* studies that demonstrated a detrimental impact of fenpropimorph at concentrations equal or above 0.2 mg L^{-1} on ERM development of *R. irregularis* (hyphal growth, spore production and mycelium architecture) (Campagnac et al. 2008, Zocco et al. 2008). Other study noticed that fenpropimorph impacted the capacity of the fungus to transport P to the host plant and a drastic decrease of alkaline phosphatase and succinate dehydrogenase activities in the ERM (Zocco et al. 2011). With the same AM fungus, an induction of oxidative stress was observed in presence of fenpropimorph at 10 mg L^{-1} (González-Guerrero et al. 2010). Thus, suggesting that this fungicide could impact other biological processes in AM fungi according to the fungicide concentration.

Glyphosate (at 2 mg L^{-1}) stimulated the GHTs emission and contact of *R. irregularis,* while no effect was noticed at lower concentrations (0.02 and 0.2 mg L^{-1}). It is not excluded that this may be related to a stress reaction to pesticide exposure. Indeed, *R. irregularis* is characterized by the production of a greater number of GHTs (de la Providencia et al. 2005). Thus, increasing the possibilities of contacts of the affected area by linking several hyphae or even facilitating root colonization. Conversely, glyphosate irrespective of the concentration used did not impact the HHM of *Gigaspora* sp. We hypothesize that the chemical signals that contribute to the emission and then contact and fusion of GHTs, are not disturbed or are less sensitive by the presence of the herbicide in the environment. Our results support a differential sensitivity of AM fungus to the presence of glyphosate. It is not excluded that these differences may be related to the sterol composition of both AM fungi as previously suggested in **Chapter I**. Azoxystrobin and fenpropimorph at 2 mg L^{-1} significantly reduced the anastomosis within the same network or between different networks of *R. irregularis*. Conversely, flutolanil and pencycuron did not impact the anastomosis formation of *R. irregularis*. Our results complement those obtained by Cardenas-Flores et al. (2011) and Novais et al. (2019a) suggesting that fungicides (e.g., fenhexamid and benomyl) at concentrations above the recommended dose calculated *in vitro* affect the anastomosis of AM fungal spores. In addition, using plant systems, Novais et al. (2019b) noticed that fenhexamid and benomyl reduced the ERM but not the anastomosis within the same mycelium, suggesting different growth strategies of ERM when exposed to fungicides. Noticeably, these results on intact mycelium confirm the diferential impact of the same fungicides on HHM process after physical injury evaluated in **Chapter 1**.

Impact of azoxystrobin on dynamics of short-term phosphorus uptake of *Rhizophagus irregularis*

In the second part of the thesis, the study was conducted under a non-destructive circulatory semi-hydroponic cultivation system. Most methods developed so far, were destructive with Pi uptake/immobilization measured in the shoot and root system (i.e., P concentration/content) or with the use of radioactive isotopes (e.g., ^{32}P, ^{33}P) (Jakobsen et al. 2021; Qin et al. 2022). Therefore, the non-destructive circulatory semi-hydroponic cultivation system, developed by Garcés-Ruiz et al. (2017) for AM fungi, was adapted to study the uptake dynamics of Pi in presence of azoxystrobin.

Boosting nutrients uptake and transport of phosphorus is an attribute of AM fungi which has been scarcely investigated in presence of fungicides (Boatman et al. 1978; Gnekow and Marschner 1989; Hale and Sanders 1982; Kling and Jakobsen 1997; Schweiger and Jakobsen 1998; Sukarno et al. 1996; Zocco et al. 2011) and, to our knowledge, never investigated with azoxystrobin. The AM fungal-colonized plants and controls were grown on perlite in containers irrigated by a flowing nutrient solution impoverished in Pi. With this system, it was possible to

evaluate the effects of azoxystrobin on the short-term Pi uptake/immobilization during a period of 72 h using potato plants associated or not to *Rhizophagus irregularis* MUCL 41833.

Azoxystrobin strongly decreased the Pi uptake by the mycorrhizal and non-mycorrhizal potato plants 30 days after application of the fungicide. In contrast, after 60 days, in absence of the fungicide, the uptake of inorganic Phosphorus (Pi) was higher in the mycorrhizal plants, while the reverse was noticed in presence of the fungicide. Indeed, azoxystrobin reduced drastically the root colonization. Our results are in good agreement with greenhouse studies that demonstrated that azoxystrobin applied as soil drench inhibit root colonization of AM fungi in sugarcane (Vuyyuru et al. 2018), maize plants (Diedhiou et al. 2004) and potato (Alarcón et al. 2022). This fungicide is also detrimental to the anastomosis process of AM fungi as shown in **Chapter 1 and 3**. This marked detrimental impact of azoxystrobin on AM fungi is probably caused by the impact on the respiratory electron transfer within mitochondria as in several pathogenic fungi (Bartlett et al. 2002; Feng, Huang, et al. 2020). However, it is not excluded that the detrimental effect of azoxystrobin may be related to the alteration of other biological important processes in the Glomeromycota.

Our results on Pi uptake and plant growth tend to support a functional alteration of the symbiosis relative to the cost in terms of nutrients exchanged with the AM fungus based on the availability of nutrients in the environment or the demand of Pi by the host plant. From the present study, it is therefore difficult to extrapolate the results to the field conditions. Further studies are therefore needed to assess the effects of fungicides on potato crops inoculated with selected AM fungi in an integrated control strategy.

In presence of azoxystrobin, a strong difference in Pi uptake by the potato plants was noticed between day 30 (first dynamics of Pi uptake) and day 60 (second dynamics of Pi uptake) with nearly no Pi uptake at day 30 conversely to day 60, where 80% and 60% of Pi were taken up by the NM and M plants, respectively. This difference was related to the dissipation half-life of this fungicide in soil/substrate. Recent findings in constructed wetlands demonstrated that AM fungi promote the degradation of fungicide tebuconazole and increase bacterial community in the envinroment (Chen et al. 2024). Therefore, further studies on the role of mycorrhizal symbiosis in pesticide degradation should be carried out to investigate the role of AM fungi in the degradation of fungicides and production of metabolites.

A key advantage of the semi-hydroponic cultivation system adapted to potato plants has the capacity to monitor and compare non-destructively the Pi uptake dynamics of mycorrhizal and non-mycorrhizal plants via the depletion of Pi in the nutrient solution flowing through the plantlets containers at different sampling points by plant development stages. However, complementary studies and improvement of the circulatory semi-hydroponic cultivation system are necessary to distinct the contribution of roots and fungus to the uptake, and thus to determine the relative contribution of each in the phosphorus uptake dynamics and accumulation. The system could be extended to analyze the uptake dynamics of other macro and micro-nutrients from their depletion in a nutrient solution, as well as to the dynamics of uptake of other crop plant at different phenological development steps (e.g., at vegetative growth or flowering) associated to specific AM strains and under non-stress as well as abiotic (e.g. salinity, drought or potential toxic elements). Other relevant research lines such as the production of secondary metabolites from pesticide degradation in mycorrhizal plants or gene expression analysis could be addressed using this method.

It is not possible to exclude that the results obtained under *in vitro* conditions may differ from those obtained on field conditions or pot experiments comparing recommended field doses. However *in vitro* culture systems offer the advantage to circumscribe the environmental conditions to a limited number of variables to investigate more precisely the impact of fungicide molecules on the plant/fungus association, while the circulatory semi-hydroponic cultivation system may sketch a more integrated picture of the impact of pesticides/molecules on this association. Both approaches are therefore complementary.

In the current agricultural context conservation agriculture (CA) is a sustainable agricultural management strategy that has been widely promoted for its ability to help mitigate the negative effects of intensive agriculture (Prestele et al. 2018). Conservation agriculture system is based on minimum soil disturbance, permanent soil cover, and crop rotation its aim is reduce erosion, enhance soil health, and rebuild soil organic matter cultivate beneficial soil life (Montgomery 2021). Studies with molecular approach demonstrated that zero tillage practices are less harmful to AM fungal diversity (Brito et al. 2012; Vishwakarma et al. 2023) and ERM (Brito et al. 2021). However intensive application of herbicides is the only viable option to deal with problem of weed management especially in the first years of establishment. The impact on AM fungi adaptation and its impact on crop yield under these agricultural systems has been poorly evaluated.

CONCLUSIONS AND PERSPECTIVES

Most agricultural crops form symbiotic associations with arbuscular mycorrhizal (AM) fungi. This has sparked interest in the potential roles of these organisms in sustainable food production. However, many agricultural practices (e.g., tillage, monoculture, application of fungicides) can affect these obligate root symbionts, explaining why most positive results obtained in pots have not been transposed to the field. The *in vitro* as well as semi-hydroponic cultivation systems used in our thesis allow us to address questions otherwise difficult to explore in pot or field conditions. This is particularly true for structures such as extraradical mycelium (ERM) which are exposed in the field to pesticides (Rivera-Becerril et al. 2017; Hage-Ahmed et al. 2019; Sweeney et al. 2022), but which are difficult to study in detail. Mechanisms such as anastomosis and hyphal healing necessitate systems allowing to monitor their development non-destructuvely in presence of stressful factors such as pesticides. Both processes are essential for the vitality of AM fungi and their functions in providing the plants with nutrients. In the present study, our focus was on conventional chemical pesticides. However, the demand for biopesticides is increasing due to demand of sustainable agriculture practices (Fenibo et al. 2022). The biopesticides are considered safe or less harmful due to their natural origin, but their effects on non-target organisms, such as AM fungi, are scarcely known and should be evaluated.

In the present study, our focus was first on the HHM and anastomosis processes in the ERM of two AM fungi (*R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331) having contrasting LHS. Then we focused our attention on the dynamics of Pi uptake of potato plants associated or not to an AM fungus grown in presence of azoxystrobin.

Four research questions were addressed:

What is the impact of different classes of fungicides on the HHM in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331, two AM fungi with different life history strategies?
We report, for the first time, the effects of fungicides on the HHM in two AM fungi (*R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331) with different LHS. Azoxystrobin (a broad-spectrum and mostly considered systemic fungicide) was the most detrimental on both fungi at concentration slightly above the recommended field dosage. In contrast, pencycuron (a contact fungicide used against *Rhizoctonia*) and flutolanil (a systemic fungicide used against Basidiomycota) did not impact any of the two AM fungi. Curiously fenpropimorph (a sterol biosynthesis inhibitor with broad-spectrum and systemic effects) only impacted *R. irregularis* by stimulating at low and inhibiting at high concentrations the emission and contact of growing hyphal tips (GHT). This observation is related to a phenomenon named hormesis. Hormesis is characterized by temporal physiological stimulation at low doses and inhibition at high doses (Jakobsen et al. 2021; Agathokleous et al. 2022). The phenomenon of hormesis, has been reported in several fungal pathogens in presence of fungicides (e.g., boscalid, carbendazim, prochloraz, kresoxim methyl, cyazofamid) (Agathokleous and Calabrese 2021), but remarkably the knowledge is limited in AM fungi. The mechanism behind the observed results remains to be assessed with special attention to:

- the apical vesicular clusters (also named spitzenkörper) in the AM hyphal tips, involved in growth, orientation, contact and fusion of hyphae. For this, a protocol using confocal microscopy as those described in Riquelme et al. (2007) could be adapted.
- the sterols content in AM fungal membrane, after extraction and analysis using GC-MS (protocol of Calonne et al. (2012)).

What is the impact of glyphosate on the HHM in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331?

Glyphosate, the world most widely applied herbicide, was shown to stimulate the emission and contact of GHTs of *R. irregularis* at high concentration (2 mg L^{-1}). Conversely, no impact was noticed on HHM of GHTs in *Gigaspora* sp. These results suggested a differential resistance/tolerance mechanism between a *Glomeraceae* and *Gigasporaceae* species to glyphosate, possibly related to the LHS of both fungi. Our results do not exclude that these differences may be related to the membrane sterol composition of both fungi and low toxicity of this herbicide in specific signaling molecules during the process of common attraction (positive tropism) in GHTs formation. The biochemical mechanisms behind the resistance/tolerance mechanism noticed remain to be unraveled in Glomeromycota. In pathogenic fungi, several mechanisms of resistance are noticed (Leconte et al. 2024):

- The appearance of point mutations in the target genes
- Increased expression of the target genes

In AM fungi, it is still difficult to predict which enzyme activity is affected by glyphosate. A global screening of the gene expression via a trancriptomic analysis of the RNA (RNA-Seq) could help in understanding which physiological metabolism is impacted by glyphosate.

- Increased expression of genes encoding efflux pumps.

Efflux pumps localized in plasma membrane are essentials in eucaryotes to export toxins outside cells and their activation was demonstrated in pathogenic fungi to export fungicides (Cannon et al. 2009; Kretschmer et al. 2009; Rizzo et al. 2019). Among them, two classes are mainly involved in the export of toxins: the ATP binding cassette and the major facilitator superfamily (Villani et al. 2016). Their activitation could lead to a resistance against pesticides or reduce the fungal sensitivity to this molecule. For this, efflux pumps modulators could be used, as described in the recent published paper of Leconte et al. (2024) on *Venturia inaequalis*. Nonetheless, the described protocole would require several adaptations to be experimented on AM fungi. An experiment on the spore germination (Buysens et al. 2015) in presence of efflux pumps modulators and glyphosate could be first tested.

How does fungicides with contrasting mode of action affect the anastomosis process within and between different networks of *R. irregularis* MUCL 41833?

We report, for the first time, the direct effects of fungicides on hyphal anastomosis between different networks in intact mycelium of the same strain of *R. irregularis*, complementing results already obtained on anastomoses between hyphae within the same mycelial network (Cardenas-Flores et al. 2011; de Novais et al. 2019a,b). The effects vary with fungicide and concentration and confirm the results obtained above, showing that the broad-spectrum fungicides azoxystrobin and fenpropimorph are detrimental at high concentration (2 mg L^{-1}). Obviously, the impact of fungicides on AM fungi under *in vitro* culture conditions can only partially reflect field situations, where edaphic factors (e.g., soil physico-chemistry, microbial populations, and long-term use of fungicides) determine fungicide degradation. Therefore, both approaches (field and *in vitro*) should be conducted in parallel. Our results does not exclude that fungicides may affect the anastomosis process by altering the hyphal tips of AM fungi and may thus affect the ability of AM fungi to develop mycelial networks in soil impacting nutrients reallocation within the networks and to the plant. Indeed, recent studies demonstrated that

agricultural soils contaminated with pesticides residues have long-term negative effects on AM fungi presence and functioning (Riedo et al. 2021; Edlinger et al. 2022). Molecular approaches considerably improved the identification and traceability of AM fungi in the field under long term pesticide exposure in agricultural soils has been scarcely studied. Currently, is little known about AM fungi species diversity in colonized roots of crop plants, particularly under long-term pesticide application. Molecular tools based on AM fungi-specific primers and high-throughput sequencing generate knowledge about the effects of pesticides on AM fungi colonization in crops and allows the traceability of a specific species. Thus, the molecular tools will provide more information about how AM fungi diversity is impacted by the interactions between pesticide application and agricultural practices in long-term field experiments.

Is the dynamics of Pi uptake by potato plants grown in presence of azoxystrobin more efficient when associated to *R. irregularis* MUCL 41833 than in absence of the fungus?

We demonstrated that azoxystrobin decreased Pi uptake by potato plants and impacted plant development as well as root colonization by the fungus. Curiously, non-mycorrhizal plants were less affected by azoxystrobin suggesting a direct effect of azoxystrobin on the AM fungus and an indirect effect on the plant-fungus associates via an imbalance between the Pi transported from the AM fungus to the plant in rule of C transported from the host to the AM fungus. Therefore, our results confirm the direct and indirect effects of fungicides on AM fungi as suggested in previous studies (Buysens et al. 2015; Hage-Ahmed et al. 2019). Although it is difficult to extrapolate our results to field situations, fungicide toxicity on crops inoculated with the generalist AM fungus *R. irregularis* deserves to be explored. Particularly, new research lines as biodegradation of agricultural fungicides in presence of AM fungi must be addressed. AM fungi protect host plants against diverse biotic and abiotic stresses and promote biodegradation of pesticides. For instance, the transcriptome sequencing elucidates molecular processes such as, zinc finger protein, intracellular/extracellular enzymes, structural proteins, anti-stress/anti-disease protein which are asociated with gene expression of AM fungus involved in the process of pesticide degradation (Song et al. 2016). Nowadays, high-throughput messenger RNA (mRNA) sequencing (RNA-Seq) technology can be employed to study genome wide gene expression in AM fungi (Venice et al. 2020). This transcriptomic analysis would help our understanding on the metabolic pathways affected by pesticides. For instance, azoxystrobin is known to affect the respiration metabolism in pathogenic fungi. A transcriptomic analysis could highlight changes in the gene expression of enzyme located on the respiratory electron transfer chain, namely, complex III [quinone outside (Qo) portion] in the mitochondrion. Future studies need to be conducted to screen the gene expression or molecular mechanisms of species of AM fungi phylogenically distant under pesticide exposure and pesticide degradation.

Further experiments are also required to evaluate, under microcosm field trials, whether azoxystrobin used against *Rh. solani* in potato crops can decrease the beneficial effect of selected AM strains on plant Pi accumulation and growth.

In conclusion, we discovered that pesticides with different modes of action and concentrations differentially affect anastomosis and HHM of two phylogenically-distant AM fungi. A detrimental effect on HHM of *Gigaspora* sp. and *R. irregularis*. Azoxystrobin at 2 mg L^{-1} also negatively impacted the anastomosis formation within and between networks of *R. irregularis*. Curiously fenpropimorph differentially impacted the HHM of *R. irregularis* (stimulating at low and inhibiting at high concentration GHTs emission and contact) and no effect was observed on *Gigaspora* sp. Fenpropimorph at 2 mg L^{-1} decreased the anastomosis formation within and

between networks of *R. irregularis*. The two other fungicides, pencycuron and flutolanil, had no effects on HHM and anastomosis. Additionally, glyphosate at 2 mg L^{-1}, only stimulated the emission and contact of growing hyphal tips (GHT) during HHM in *R. irregularis*, instead no impact was noticed in *Gigaspora* sp. Finally, we confirm the harmful impact of azoxystrobin on potato plants colonized and not colonized by *R. irregularis* MUCL 41833. The mycorrhizal potato plants (P transfer, root colonization and biomass) were more affected by azoxystrobin than those growing in absence of the fungicide. These results suggest a direct effect of azoxystrobin on the fungus (its capacity to absorb and transport Pi to the plant) and an indirect effect via a probable imbalance between the Pi transported from the AM fungus to the plant in rule of C transported from the host to the AM fungus.

Our findings suggest that pesticides affect the HHM and anastomosis in AM fungi with contrasting LHS possibly by altering the hyphal tips and may thus affect the capacity of AM fungi to survive and develop mycelial networks in soil. The mechanisms remain to be discovered in view of an integrated management of chemicals to control fungal pathogens without harming the beneficial effects of AM fungi.

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ANNEX I

Modified Strullu-Romand (MSR) medium

Solution 1: Macroelements

MgSO4.7H2O 73.90 g L^{-1}

KNO3 7.60 g L^{-1}

KCl 6,50 g L^{-1}

KH2PO4 0.41 g L^{-1}

Solution 2: Calcium Nitrate

Ca (NO3)2.4H_2O 35.9 g L^{-1}

Solution 3: Vitamines

Panthotenate Ca 0.18 g L^{-1}

Biotine (solution: 1 ml/500 ml (9 mg/100 ml)) 0.18*10-3 g L^{-1}

Nicotinic acid 0.20 g L^{-1}

Pyridoxine 0.18 g L^{-1}

Thiamine 0.20 g L^{-1}

Cyanocobalamine 0.08 g L^{-1}

Solution 4: Na Fe EDTA

Na Fe EDTA 1.60 g L^{-1}

Solution 5: Microelements

1. Dissolve 1.225 g of $MnSO_4.4H_2O$ (0.93 g of $MnSO_4.H_2O$) in 100 ml pure distilled water

2. Dissolve 0.14 g of $ZnSO_4.7H_2O$ in 100 ml pure distilled water

3. Dissolve 0.925 g of H_3BO_3 in 100 ml pure distilled water

4. Mix 1+2+3

5. Weigh 1.1 g of $CuSO_4.5H_2O$ and dissolve in 50 ml pure distilled water

6. Pipet 5 ml to sol'n 1+2+3

7. Dissolve 0.12 g of $Na_2MoO_4.2H_2O$ in 100 ml pure distilled water

8. When it is completely dissolved, pipet 1 ml to sol'n 1+2+3

9. Dissolve 1,7 g of (NH4)6Mo7O24.4H2O in 100 ml pure distilled water

10. When it is completely dissolved, pipet 1ml in sol'n 1+2+3

11. Adjust the volume of sol'n 1+2+3 to 500 ml

To prepare a 1 L volume of solid medium

10 ml of solution 1 (macroelements)

10 ml of solution 2 (Ca $(NO_3)_2.4H_20$)

5 ml of solution 4 (Na Fe EDTA)

1 ml of solution 5 (microelements)

5 ml of solution 3 (vitamins)

10 g of sucrose

Dissolve completely and adjust the volume to 1 L (H_2O Type II)

Adjust the pH to 5.5

Add gelling agent (3 g L^{-1})

Autoclave for 15 minutes at 121°C

With powder

To prepare 1 L

0.594 g powder MSR

0.256 g Ca (NO3)2

10 g of sucrose

Adjust the pH to 5.5

ANNEX II

Murashige and Skoog (MS) medium

Microelements	**mg L^{-1}**
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Macroelements	**mg L^{-1}**
$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Vitamins	**mg L^{-1}**
Glycine	2.00
myo-Inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10

MS medium was solidified with 4.2 g L^{-1}Gelrite™ and supplemented with 20 g L^{-1}sucrose.

ANNEX III

Hoagland solution (90% P-impoverished solution Phosphorus = 6.245 mg L^{-1}) (Hoagland and Arnon, 1950).

Compound	mg L^{-1}
NH_4NO_3	80
$Ca(NO_3)_2 \cdot 4H_2O$	826
KNO_3	357
KCl	45.1
K_2SO_4	105.4
KNO_3	50
KH_2PO_4	27.4
$MgSO_4$	120.4
$MnSO_4 \cdot H_2O$	0.5
H_3BO_3	1.4
$CuSO_4 \cdot 5H_2O$	0.2
$(NH_4)6Mo_7O_2 \cdot 4H_2O$	0.1
$ZnSO_4 \cdot 7H_2O$	0.6
Fe-EDTA	19

The pH of the solution was adjusted to 5.6 ± 0.2 before use.